THE IMMUNOBIOLOGY OF GRAFT VERSUS HOST DISEASE AND ITS ATTEMPTED PREVENTION USING NATURALLY OCCURRING SUPPRESSOR FACTORS

BY

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
UNIVERSITY OF FLORIDA

1983

ACKNOWLEDGEMENTS

I would like to express my appreciation to the many people who helped me complete this dissertation.

To my parents, without whose help and understanding I would never have made it this far, I extend my gratitude.

I would like to express my deep thanks to Dr. Ammon Peck for his help and guidance through this work, especially his willingness to give me a free hand performing these experiments, related or unrelated to this dissertation which allowed me to satisfy my insatiable curiosity. In addition, his friendship is warmly remembered.

Appreciation is also given to Drs. Ward Wakeland and Art Kimura for their discussions and insights about the immunogenetics of the mouse system. Additional thanks are given to Dr. Wakeland and Vicki Henson for their preparation of monoclonal antibodies and Dr. Kimura for the column chromatography.

Thanks are also given to Dr. Paul Klein for whom a generous supply of materials allowed me to get through the first year of this project and Drs. Richard Smith and Shiro Shimuzu from whom I was able to acquire enough interleukin 2 in order to maintain and expand the cytotoxic T cells which were important to this study. Thanks are also extended to Dr. Micheal Norcross for helping with the photography.

Thanks are also extended to Drs. Roy Weiner and Noel McClaren who shared their help and time while participating on my advisory committee.

Another round of appreciation is given to those people who helped with the mice: Mr. Lucetta for breeding the necessary mice for most of this work, Dr. Bobby Collins for his preparation of the histology of the various tissues, as well as Steve Noga for his help in interpreting the various histological and pathological sections which were reported.

Thanks are also given to Dan Cook and Dr. Jian Xiang Huang who helped me with the word processing of this work. Without their help this epic manuscript would have cost a fortune to produce.

A special round of gratitude is given to the excellent technical help of Laura Prall, Jean Mahlman and Mary Ann Searle who helped me with the arduous and time consuming tasks such as preparations of various materials which allowed me the necessary time to plan further GVH conquests. Thanks also go to Vi Sudipong for her participation with the minor histocompatibility work during the summer of 1982.

Finally, a word of gratitude is given to my fellow graduate students: Vicki Henson, Ted Hall, Kim Peeler and Dave Shaut who had to share in my moods when things were not correct.

TABLE OF CONTENTS

CONTENTS—Continued

CONTENTS—Continued

LIST OF TABLES

Table page

TABLES--Continued

TABLES—Continued

TABLES—Continued

LIST OF FIGURES

FIGURES--Continued

FIGURES—Continued

Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

THE IMMUNOBIOLOGY OF GRAFT VERSUS HOST DISEASE AND ITS ATTEMPTED PREVENTION USING NATURALLY OCCURRING SUPPRESSOR SUBSTANCES.

By

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August 1983

Chairman: Dr. Ammon B. Peck
Major Department: Pathology

A model for the murine graft versus host (GVH) reaction has been developed in order to examine the potential value of naturally occurring suppressor cells and factors in preventing lethal disease. GVH reactivity was induced in sublethally irradiated adult mice by reconstituting the hosts with allogeneic splenocytes. Nonsuppressed host/donor combinations with genetic differences at H-2 died within 14 days. Combinations with I and K/D genetic differences showed longer survival times, while mismatches involving non-H-2

generally had no mortality. T lymphocytes specifically reactive against host tissue antigens could be recovered from diseased organs such as spleen, lymph nodes and liver. Based on cell surface phenotype and in vitro reactivity, two distinct populations of cells were recovered: one proliferated against class II molecules, the other lysed cells expressing class I molecules. This situation proved different from reactivity profiles of cells activated in mixed lymphocyte reactions.

The presence of cytotoxic T cells correlated with GVHD mortality. The presence of large numbers of CTLs was common in H-2 GVH mismatches and no doubt contributed to the rapid death. In contrast, only weak, but transient CTL activity was found in mismatches involving class II antigens; however, the recovered cells could be expanded in vitro with interleukin 2. The resulting cells exhibited cytotoxic activity against both donor and host.

Attempts were made to prevent GVHD using cells from the spleens of newborn mice known to contain naturally occurring suppressor cell populations. Unexpectedly, only certain strains of newborn mice possessed the capacity to suppress lethal GVHD. The genetics of suppression by newborn spleen cells suggested two restrictions: first, the newborn spleen cells apparently must express the strongly stimulating Mls

antigens, and second newborn spleen and adult donor cells must be histocompatible at a genetic region telomeric to I-A. No T cell reactivity could be detected up to 60 days in mice showing long term survival. During this time, these mice remained chimeras. After 60 days postengraftment, the cell and reactive phenotype of these mice returned to that of the host.

INTRODUCTION

1.1 The Graft Versus Host Reaction

The graft versus host reaction (GVH) is a unique model to study immunoregulation in that the entire sequence of an immune response (initiation, differentiation, a multifaceted effector phase) and final control is represented. An extensive body of knowledge concerning the initial events of antigenic recognition, cell types involved, cell differentiation, and final expression of immunocompetence during the GVH reaction already exists (reviewed in references 1,2). However, there is comparatively little understanding of the complex mechanisms and cellular interactions regulating the course of the on going immune reaction(s).

The most common forms of GVH reactions are runt disease, secondary disease, parabiosis and F_1 hybrid disease. Runt disease occurs when mature competent allogeneic cells are injected into an immunoincompetent newborn. Growth of the newborn is inhibited and quite frequently the animal dies manifesting severe diarrhea, dermititis, hepatomegaly, and splenomegaly.

Secondary disease is seen in those adult animals who have been immunocompromised by either drugs or irradiation and have been reconstituted with allogeneic stem cells with the following results: diarrhea, dermatitis, renal lesions (immune complex deposition), hemolytic anemia and hepato and splenomegaly.

Parabiotic intoxication occurs when two allogeneic adult mice have been surgically treated so that they share a common circulation and their lymphocytes are free to attack the other animal.

Hybrid disease happens when a F_1 hybrid has been injected with cells from the parental strain. The laws of transplantation (See Figure 1) state that when two histocompatible homozygous animals mate, the F_1 progeny possess the histocompatibility antigens of both parents and are able to tolerate grafts from either parent, while the parent rejects grafts from the progeny because of the histoincompatible antigens. Thus the donor cells recognize

Figure 1. The laws of transplantation. Each inbred mouse strain is homozygous for the H-2 complex. Both sets of products are expressed in the heterozygous F_1 hybrids. These mice therefore produce H-2 antigens that stimulate an immune response in either parental strain, whereas, neither parental strain express H-2 antigens foreign to the F_1 hybrid.

1) Grafts within an inbred strain (syngeneic grafts) succeed.

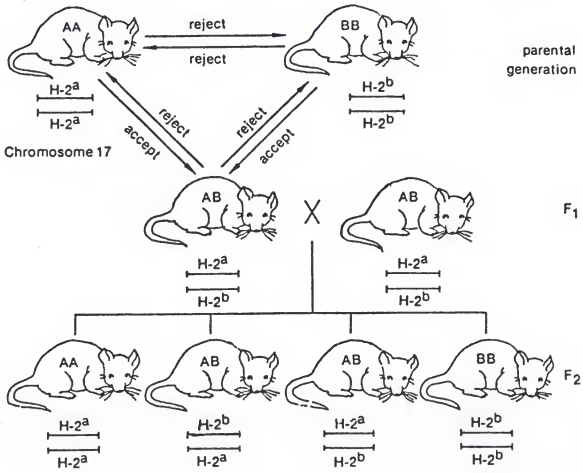
2) Grafts between different inbred strains (allogeneic grafts) fail.

3) Grafts from either inbred parent strain to the F_1 hybrid succeed but grafts in the reverse direction fail.

4) Grafts from F_2 or subsequent F generations to F_1 hybrids succeed.

5) Grafts from either inbred parent strain succeed in some members of an F_2 generation but fail in others. Also, grafts from one inbred strain succeed in some members and fail in others of a backcross produced by crossing the F_1 hybrid to the opposite parent strain.

Taken from Klein (3) and Hood et al. (4)


AA
reject
reject
BB
parental generation
H-2a
H-2a
H-2b
H-2b
Chromosome 17
reject
accept
reject
accept
AB
X
AB
F1
H-2a
H-2b
H-2a
H-2b
AA
AB
AB
BB
F2
H-2a
H-2a
H-2b
H-2a
H-2a
H-2b
H-2b
H-2b

the F_1 being foreign, but the F_1 can not normally react to the donor cells because they see those cells as being self. The advantage of this system is that the recipient possesses an intact immune system.

Currently GVH reactions are studied either systemically or locally. Both sets of reactions have been reviewed by Grebe and Streilein (2) and can be summarized as follows:

A) Systemic

Inhibition of syngeneic hematopoeitic colony forming units: Irradiated F_1 mice are given parental lymphoid cells along with syngeneic bone marrow cells. The amount of erythroid cell growth is assayed by the amount of ^{59}Fe that is incorporated into the spleen. The less ^{59}Fe incorporated, the greater the immune response was towards the host.

The Simonsen spleen index assay: This assay utilizies the fact that when allogeneic cells are injected into a recipient the spleen enlarges in response to the allogeneic challenge.

The phagocytic index: This is an indirect test used in F_1 animals 2 weeks after the parental cells are injected. Because of the increased lymphoid activity, colloidal carbon is cleared from the system in a much shorter time.

The focal periportal infiltration method: This system deals with enumerating the number of foci seen in the F_1 liver after parental cells are injected.

The splenic explant assay: Single cell suspensions of parental cells are placed over diced F_1 spleens. The culture is allowed to procede 5 days and then the culture is examined for increased physical masses.

B) Local

The epidermolytic reaction: sensitized lymph node cells are injected intracutaneously into F_1 animals. The blood cells of the host are destroyed followed by nonspecific vascular destruction in the skin with noticeable epidermal necrosis.

Intraocular or intrarenal GVH: allogeneic cells are injected either into the anterior chamber of the eye or under the renal capsule; within a short period of time immune reactions cause gross morphological changes in these organs.

The popliteal lymph node assay: this assay is based on the same theory as the Simonsen spleen index except reactive allogeneic cells are injected into the animals footpad with subsequent measurements of the popliteal lymph node 1 to 2 weeks later.

Often, the final result of GVH is the death of the host. At times, however, the host may survive GVH, suggesting the development of "tolerance". The mechanism(s) underlying tolerance, both host towards the graft as well as graft toward the host, remain only speculative, but may result from

1] blocking factors, such as antibody possessing the potential to bind to antigenic sites of the host cells (5,6)
2] suppressor cells, which inhibit the regulating T helper cells (7,8) or
3] destruction of the stimulating components of the host, presumably the lymphoid associated cells (9,10).

The lymphoid cells of the host have been implicated in the histological and clinical manifestations of GVH in two ways. First, these cells no doubt provide the immunogenic stimuli for GVH through the trapping the donor cells within the lymphoid organs, thereby stimulating the donor cells into growth (11,12). Second, the host cells, while under GVH siege, may be nonspecifically stimulated to grow (13,14). These proliferating host cells could either be target cells or they could become autoimmune to their self via an allogeneic effect mechanism (15,16) or via an inflammatory response.

Attempts to alter GVH reactions have intensified with the development of human transplantation systems.

Pharmacological agents have seen a major trend towards limiting GVH reactions. Cyclophosphamide and the cortical steroids have been tried clinically with less than expected results (17,18,19). A major problem with such drugs is that these agents nonspecifically depress the immune system, thereby promoting other deleterious effects such as increased risk of infections and lymphomas. Cyclosporin A has recently become a center of a pharmacological approach to limit GVH, but it too may be nonspecifically immunosuppressive (20,21,22).

Another approach to this question has been to stimulate suppressor cells using the plant lectin, concanavalin A (Con A)(23,24,25,26). Certain doses of Con A are known to be mitogenic while other doses are known to be suppressive (25). Unfortunately, this drug also has toxic side effects and therefore could be hard to judge adequate dosages for any given individual.

Treatment of donor cells with various antibodies and complement has been another effort which has been examined (27). Until quite recently, the only antisera which had been employed in humans was rabbit anti-lymphocyte antisera. The hope here was to eliminate immunocompetent T cells (while leaving virgin bone marrow cells intact) before they have had a chance to become stimulated. Unfortunately, this regimen has had only limited success which could be due to

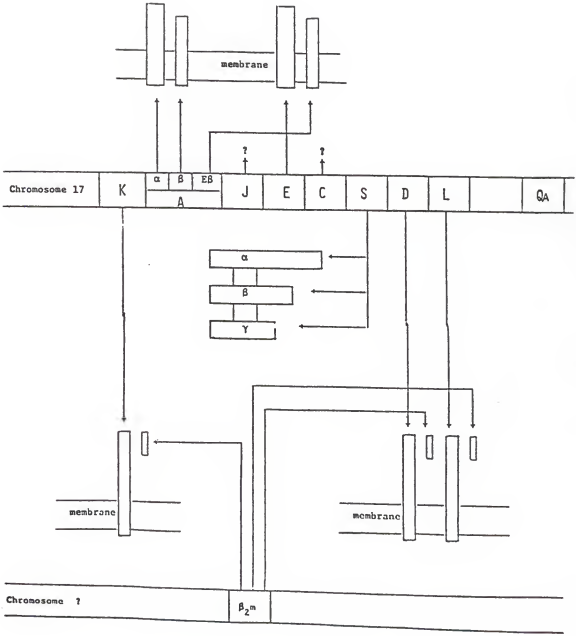
the poor specificity and titer of the antisera used. Perhaps the development of appropriate monoclonal antibodies might circumvent this problem (28,29). In any event, there is little disagreement on the need for better methods to achieve the final goal of tolerance.

1.2 The In Vitro Measurement of the GVH Reaction

The murine major histocompatibility complex (H-2) consists of a number of immunologically important loci: K,D,S and I (Figure 2). The K and D regions code for molecules which direct lympholysis; the S region produces serum proteins such as the fourth component of complement, while the I region genes and associated antigens have been reported to be involved in a large number of immunological phenomena such as T-B cell cooperation (30), antigen presentation by macrophage (31), helper factor (32,33), suppressor factor (34,35) and blastogenesis in mixed lymphocyte reactions, MLR (36).

Genetic disparity between donor and recipient which apparently controls initiation of GVH is determined primarily within the major histocompatibility complex (MHC) of the species, in particular the I region (in the mouse) or its equivalent in other species. Representative data

Figure 2. The major histocompatibilty complex of the mouse. This schematic drawing of the MHC of the mouse (H-2). The H-2 is composed of several loci, as denoted on the figure. The MHC gene products is composed of several types of molecules. Class I molecules such as: K, D and L molecules are single 45,000 molecular weight proteins noncovalently associated with beta two microglobulin molecules. The class II molecules: I-A and I-E consist of two noncovalently linked proteins of 35,000 and 28,000 daltons. Finally, the class III molecule encoded by the S region describes a serum component C'4 which is a component of the complement series of protein.


membrane
?
?
Chromosome 17
K
α
β
EB
A
J
E
C
S
D
L
QA
α
β
γ
membrane
membrane
Chromosome ?
β_2^m

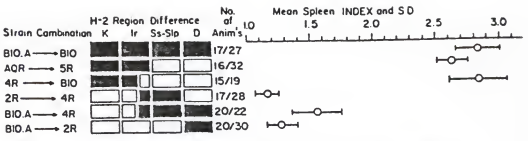
revealing the importance of the I region, taken from Klein and Park (37), is presented in Figure 3.

Even though K and D region differences produce some splenic enlargement as evidenced by an increased splenic index of 1.5, this increase of 1.5 probably represents just the homing in of the injected spleen cells into the host spleen without any significant reaction. But when I region differences occur, the spleen doubles or triples in size due to the development of a significant reaction.

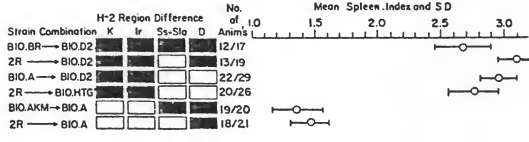
The in vitro measurement of GVH is normally performed using the mixed lymphocyte reaction (MLR) (38,39,40,41). This test measures the proliferative phase of T cell activation following recognition of allogeneic antigens. Representative data comparing MLR and GVH are presented in Table 1 (37).

In vitro studies using the MLR as a model for GVH have provided extensive insight into T cell recognition, differentiation, and cell interactions. Activation of T lymphocytes by MHC incompatibility results in increased DNA, RNA and protein synthesis, increased energy utilization and increased size (42,43,44). During this stage, the T cells proliferate and differentiate into either the proliferating helper T cell or the poised precursor of the cytotoxic T cell. The poised cytotoxic T cell further differentiates to

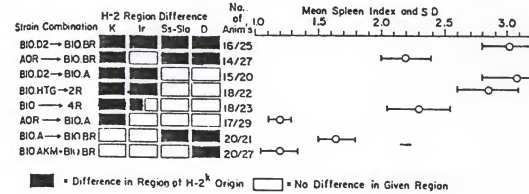
Figure 3. Graft versus host reactions across various H-2 differences. This figure represents splenomegaly indices of various mice undergoing graft versus host reactions. Taken from Klein and Park (37).



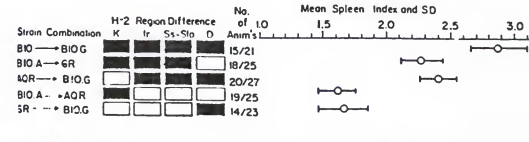
GVHR across *H-2*^{b} differences. Number of animals: control/experimental.



GVHR across *H-2*^{d} differences. Number of animals: control/experimental.



GVHR across *H-2*^{k} differences. Number of animals: control/experimental.



GVHR across *H-2*^{q} differences. Number of animals: control/experimental.

Table 1.
Strenth of MLR and GVHR across various regions of the H-2.

H-2 region difference	MLR		GVHR	
	average ratio of stimulation	range	mean spleen index	range
K	2.0	0.7-4.7	1.4	1.2-1.6
D	1.8	0.8-5.4	1.4	1.2-1.7
I-A	6.4	3.7-9.2	1.8	1.8
I-B+S	2.7	1.1-4.4	1.5	1.1-1.8
I+S	5.8	2.7-12.8	2.6	2.6
K+I	6.6	3.2-18.3	2.8	2.6-3.1
D+S	2.0	0.7-4.7	1.5	1.3-1.6
K+D	3.4	3.0-3.8	1.8	1.1-2.5
K+S+D	3.3	1.5-8.6	2.3	2.2-2.4
K+I+S+D	7.2	1.2-33.6	2.8	2.4-3.1

Taken from Klein (3)

the mature killer cell after receiving the help signal from the helper T cell.

Killer T cells were originally characterized as possessing the Lyt 2 and Lyt 3 antigens by negatively selecting T cells with anti-Lyt 1 antisera and complement (45,46). Recently, killer cells possessing the Lyt 1 phenotype have been described (47). The discrepancy in 2 killer T cell phenotypes may be due to the sensitivity in the antisera used. Originally anti-Lyt 1 antibody and complement killed the high density Lyt 1+ cells so that weakly positive Lyt 1+ cells still are present. Only through sensitive immunoflourescent techniques can one observe the low density Lyt 1+ cells (48).

Helper T cells reacting against whole haplotype, I region, or non-MHC antigens have been shown to bear only Lyt 1 antigens (49,50). In contrast, T cells responding against K/D differences have been found to be Lyt 1,2,3+ cells (51). This as well may be an artifact with the Lyt 1,2,3+ cells being precursors to the T helper cells, Lyt 1+.

The blast cells do not remain in this activated mode for long as they revert to small lymphocytes capable of exhibiting memory (52). These cells can be restimulated by the initial antigen in a secondary manner, with the peak response occurring on day 2 of culture as opposed to days 5 and 6 found in a primary reaction. The primed lymphocyte

test (PLT) utilizies this concept of *in vitro* restimulation to specifically quantitate similar histocompatible antigens on third party cells (53,54). This procedure has only been developed in the last few years; however, the principles of these *in vitro* restimulations offer much potential in probing some basic questions of vital importance in immunobiology such as the activation of memory cells, the differences between primary and secondary stimulation, the specificity of the response towards antigens, and the number of different cell subpopulations responding towards one antigen. All these activities have been observed at the gross level, but the actions of single clonally expanded cells have only been recently approached. By selectively expanding blast cell cultures one may be able to examine the fine specific activities of individual cells which were not available previously.

T cell clones have been established in various laboratories using a variety of different strains of mice as the source of responder cells. Essentially two types of functional clones have been found. One type of clone is cytotoxic towards the stimulating cell only when that specific antigen bearing cell and some exogenous helper factor(s) are present (55,56,57,58,59). Since these clones carry out this cytotoxic effector function, it is presumed that these cells originated from a cytotoxic T cell

possessing Lyt 2,3 antigens. These cells may be maintained in culture for weeks solely by the presence of the exogenous factors even without the initial stimulating antigen. Interleukin 2 (IL 2) is one of those exogenous factors; it is a 30,000 to 35,000 dalton protein derived from splenic lymphocyte cultures stimulated by T cell mitogens Con A or phytohemagglutinin (60,61,62,63,64), from other MLR reacting supernates (65,66,67) or from clones of T helper cells (58,59,68).

The second type of clone undergoes proliferation when presented with the specific priming antigen, e.g., histoincompatible cells, or soluble antigens, but does not exhibit any cytotoxic potential. No exogenous factors are apparently needed by these clones. However, these cells can become addicted to IL 2 and can lose their specificity and ability to respond to antigen. A few of these clones have been shown to produce factors which activate the cytotoxic T cell clones in vitro (58,59), strongly suggesting that these clones are members of the helper T cell class. Helper T cell clones have also been shown to function in vivo by increasing antibody formation towards T cell dependent antigens, e.g., sheep red blood cells and horse red blood cells in nude mice (69).

In addition to nonspecific helper factors such as IL 2, T cells have been claimed to produce antigen specific

factors which stimulate naive B cells into making specific antibodies towards a given antigen. Some of these factors bind to the antigen directly, possess Ia determinants, and have a molecular weight of 35,000 daltons. These factors when isolated from T cells can direct the B cells into antibody secretion (70,71,72,73). Whether a given T cell secretes both IL 2 and antigen specific helper factor is not known.

The majority of cytotoxic clones which have been established have activity against specific membrane moieties, like H-2K or H-Y antigens (74,75,76). In contrast, the majority of proliferative helper T cell clones have activity against non-MHC antigens (77,78), although a few have been selected with activity against H-2 I region gene products (Ia) (79,80).

One of the problems with long term lymphocyte cultures and clones has been the frequent and regular appearance of a crisis phase in the growth pattern of these cells (68). After 3 to 6 months in culture the majority of the responding cells die; the remaining cells replicate at a slower pace than they did before crisis set in. This crisis period lasts for about 3 weeks, after which time the cells may once again grow. Thus, long term cultured cells can be used for analysis only during the limited periods between crises.

1.3 Controlling the Activities of T Cells In Vitro

Alpha fetoprotein (AFP), a normal component of fetal and newborn sera, has been shown in both human and murine systems to exert selective suppressive effects on various functions of T lymphocytes, including T cell dependent antibody synthesis, T cell mitogenic responsiveness, and T cell mediated allogeneic reactivity (81,82,83,84,85,86). In addition, recent reports have revealed that under certain circumstances AFP may exert a supportive influence on *in vitro* cell growth with one manifestation being the *in vitro* induction of suppressor T cells (83).

Analysis of the impact exerted by AFP on the recognition and subsequent proliferation of T lymphocytes reacting in MLR against defined histocompatibility alloantigens has revealed a highly selective activity in the suppression of lymphocyte responses (81,82). In general, AFP inhibits Lyt 1+ T blast cells reacting against I region structures, including reactions against Mls locus products, but fails to inhibit Lyt 2+ cells stimulated by K/D alloantigens (81). Thus, it seems clear that AFP exerts its suppressive activity in MLR via selective interference with I region triggering systems. However, AFP also suppresses the effector phase of the T cell mediated cytotoxic reaction, thus suggesting a broader spectrum of regulatory

activity. For example, AFP, when present during the primary activation phase of T cell responses, not only suppresses the subsequent *in vitro* generation of effective cytotoxic T cells in strain combinations with I plus K/D region differences, but also in strain combinations possessing only K/D region differences where the proliferative phase was unaffected. If AFP interferes only with I region triggering, then it would have been expected that at least in reactions directed against isolated MHC SD region associated gene products not only the proliferative but also the cytotoxic phase would have remained refractive to the suppressive activity of AFP. More recently, studies by Peck *et al.* (87) have shown that AFP acts on the stimulating cell population known to initiate T cell reactivity. Furthermore, the T cell subpopulation which is refractive to AFP could be shown to exert suppression of normal primary responses. This fact suggests that AFP may be a physiological substance which could be used to control specifically the immune response in GVH reactions.

1.4 Suppressor Cells

A subpopulation of cells has been described which resides in the spleens of the newborn mouse which possesses a short lived antigen nonspecific suppressor activity for

immune reactions (88,89,90). This suppressor activity is not associated with spleen T cell populations as it is absent from purified spleen T lymphocytes, resistant to treatment with anti-I-J and anti-Ia antisera, present in the spleens of T cell deficient nude mice. In addition, suppressor activity is not due to macrophages since the effector cells fail to adhere to either plastic or Helix pomatia lectin coated plates. Natural killer cells can also be excluded since the suppressor activity fails to pass through Ig anti-Ig coated columns (Peck, unpublished results). In addition, the cells from those animals are capable of producing a soluble factor which is capable of inhibiting adult cell responses both MLR and CML.

Another set of suppressor cells has been postulated (2) to be responsible for the inability to transfer GVH from a host animal undergoing GVH reaction to a second normal animal even when both animals are genetically identical. Perhaps that time required for secondary reactivity to establish itself in the second host may permit an antigen specific suppressor cell to develop.

Both of these activities need to be further investigated as physiological entities to suppress immune responsiveness.

1.5 The Rationale for These Experiments

GVH reactions have been studied in mice in a wide variety of ways, ranging from local footpad swelling assays to splenomegaly studies in newborn hosts. Unfortunately, the majority of these studies are not homologous to the situation seen in human GVH following bone marrow transplantation. Studies dealing with the homologous situations in adult mice undergoing GVH can be classified into two main categories: first, those which have dealt with the histopathological lesions, and second, those studying the ability of cytotoxic T cells to develop using an entire H-2 mismatch. The purpose of this research was to develop a model to study GVH reactions resulting from specific H-2 and non-H-2 incompatibilities between donor and host in order to study the genetic control of T lymphocyte reactivity in GVH disease, then determine the feasibility to control this reactivity with physiological pregnancy associated substances.

MATERIALS AND METHODS

2.1 Animals

Inbred lines of mice used in these studies and maintained in the Department of Pathology, University of Florida, include A/J, AKR/J, A.TFR5, A.TL, BALB/cJ, BALB/c^{dm2}, B10.A, B10.AQR, B10.A(2R), B10.A(3R), B10.A(4R), B10.A(5R), B10.BR, B10.BUA16, B10.CHA2, B10.D2, B10.F, B10.HTT, B10.M, B10.M(17R), B10.MBR, B10.PL, B10.RIII, B10.S(7R), B10.S(9R), B10.SM, B10.T(6R), B10.Q, B10.TL, CBA/CaH, CBA/J, C3H/HeJ, C57BL/6J (B6), C57BL/6^{bml}, C57BL/10 (B10), C57BR, C58, D1.C, DBA/1J, DBA/2J, PL/J, NZB, RF/J, SEA/J, SEC/J, SJL/J, SM/J and SWR/J. Breeding pairs of B10.GD, B10.RIII, B10.S(7R) and B10.S(9R) were originally provided by Dr. Duncan, Department of Cell Biology, University of Texas, Dallas, Texas, while B10.AQR and B10.TL were obtained from Dr. Shreffler, Department of Genetics, Washington University, St. Louis,

Missouri. B10 males were bred to B10.BR females, A.TL males bred to B10.MBR females, and B10.T(6R) males bred to B10.AQR females provided the F_1 hybrids, (B10 x B10.BR)F_1, (A.TL x B10.MBR)F_1, and (B10.T(6R) x B10.AQR)F_1. Both male and female mice ranging in age from 6 to 24 weeks were used; however, mice were sex matched when used for various experiments.

2.2 Antisera

Monoclonal anti-Lyt, anti-Thy-1 and anti-I-A^k antibodies were obtained from cell lines 53-7.313 (anti-Lyt 1), 53-6.72 (anti-Lyt 2), HO-13-4 (anti-Thy-1.2) and 10-3.6.2 (anti-I-A^k) generously provided by Dr. Ledbettor and Dr. Herzenberg via the Salk Institute Cell Distribution Center, San Diego, California, while anti-K^d (B 312) and anti-I-E^k (14-4-4) were provided by Dr. D. Sachs, NIH. Each cell line was grown at high concentration in RPMI 1640 supplemented with fetal bovine serum to 10%. Supernates were used undiluted.

The antibodies 10.2.16, B 312 and 14-4-4 used in this research were generated in (B6 x BALB/c)F_1 mice using the ascites approach. These antibodies were demonstrated to high cytotoxic titers and were generously provided by Dr. E. Wakeland.

Arsenilic acid conjugated anti-Lyt 1 and anti-Lyt 2 antibodies and fluorescenated rabbit anti-arsenilate antibody were obtained from Becton-Dickinson, Oxnard, California. Fluorescenated rabbit anti-mouse Ig antibody was obtained from Cappel Laboratories, Cochransville, Pennsylvania.

2.3 Complement Dependent Antibody Cytotoxicity

A two step complement dependent cytotoxicity assay was used to treat cell populations with various antisera. Spleen cells at 5.0×10^6 cells/ml were incubated with antiserum for 45 min. at room temperature. The cells were then washed, resuspended in rabbit complement (Accurate Scientific, Hicksville, New York) and reincubated at 37 C for 45 min. Cell viability was assessed by trypan blue dye exclusion.

2.4 Fluorescent Microscopic Determination of Cells

Immunofluorescent staining of lymphocytes was performed by reacting 5.0×10^6 lymphocytes with either arsenilate conjugated anti-Lyt 1 or anti-Lyt 2 antibodies for 45 min. at 4 C. The cells were washed 3 times with phosphate buffered saline (PBS) followed by an incubation with fluoresceinated rabbit anti-arsenilate antibody for 45 min

at 4 C. The cells were examined for fluorescent staining through a phase contrast microscope equipped with a Zeiss Ploem UV illuminator. Cell surface immunoglobulin was detected in a similiar manner using a one step incubation with fluorescein conjugated rabbit antimouse immunoglobulin.

2.5 Cell Preparations

Whole spleen leukocyte populations were prepared as described by Peck and Bach (91). In brief, spleens freshly removed from mice were dispersed by pressing the spleen through a wire mesh screen into PBS. Following one wash, the red blood cells were lysed in a 10 minute 0.84% ammonium chloride treatment. The resulting leukocytes were washed once and resuspended in PBS to appropriate cell concentrations.

2.6 Primary Mixed Leukocyte Reaction

Primary MLRs were carried out according to the protocol of Peck and Bach (91). Throughout the study, 60×10^6 splenic leukocytes were cultured together with 100×10^6 (2000R) stimulating whole spleen cells in 30 ml EHAA media supplemented with normal mouse serum to 0.5% (92). Cell cultures were harvested between days 7 to 10 of incubation

and examined for reactivity in secondary MLR (PLT) and cell mediated lympholysis assays (CML).

2.7 Induction of Graft Versus Host Reactivity

Forty million donor (responding) splenic leukocytes were injected intravenously via the tail vein into sublethally irradiated (650R) recipient (host) adult mice. Animals were fed on lab chow and given acidified water to drink. No major problems developed from bacterial infections.

At various time points (indicated in the text and footnotes) the recipient mice were sacrificed. Their livers, spleens, kidneys, intestines, lungs and skin were removed, fixed in formalin and embedded in paraffin for routine hemaoxylin and eosin stained histopathological study. Cells present in the spleens, lymph nodes and livers were prepared and examined for functional activities.

2.8 Primed Lymphocyte Typing Tests

Antigen activated cells were separated from small and dead cells on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, New Jersey) (d=1.077) density gradients. PLT assays were performed in round bottomed 96 well Lindbro

microtiter plates (Flow Laboratories, McLean, Virginia) as described by Peck and Wigzell (93). Cultures consisted of either 0.04×10^6 responding spleen, lymph node or liver cells cultured with 0.5×10^6 irradiated stimulating spleen cells.

At appropriate times of the secondary culture, as indicated in the Tables, 1.0 μCi tritiated thymidine (^{3}H-TdR) (Amersham, Boston, Massachusetts) in a volume of 0.02 ml was added to each well for 12 hrs. Cells were then aspirated through Whatman glass fiber filters with a multiple sample harvestor (Otto Hiller Inc., Madison, Wisconsin) and total ^{3}H-TdR incorporation was determined by liquid scintillation procedures. Data are expressed in counts per minute of the average of either duplicate or triplicate cultures. Standard deviations of the average are included.

2.9 Cell Mediated Lympholysis Assays

CML were performed according to the procedure detailed elsewhere (94). The effector cells were generated in primary MLR or GVH. Alloantigen activated cells were collected from mixed lymphocyte reactions or diseased organs, centrifuged and washed twice in medium containing newborn calf serum (Biocell Laboratories, Carson, California).

Concanavalin A (Pharmacia Fine Chemicals) stimulated spleen cells were used as the target cells. Approximately 36 hrs before the CML assay, appropriate target cell cultures containing 12 x 10^6 spleen cells in 5.0 ml of EHAA with 10% newborn calf serum were established in culture dishes. Target cells were incubated 2 hrs with 400-500 µCi $Na_2{}^{51}CrO_4$. The labeled cells were washed 3 times in fresh medium.

Cell destruction was performed in V bottomed microtiter plates (Lindbro) using various effector cell numbers plus 1.0 x 10^4 labeled target cells. Cell destruction proceeded 6 hrs at 37 C after which time the plates were centrifuged, the supernate collected and the quantity of released of ^{51}Cr determined. Percent cytotoxicity is expressed as

$$\frac{^{51}Cr \text{ released (experiment)} - {}^{51}Cr \text{ released (spont.)}}{^{51}Cr \text{ released (maximum)} - {}^{51}Cr \text{ released (maximum)}} \times 100$$

Spontaneous release ranged between 15 to 20% of the maximum release.

2.10 Preparation of Purified Interleukin 2

Interleukin 2 was produced by culturing EL-4 G-12 cells, an azogaunine resistant EL-4 (T lymphoma cell line) with 8 µg/ml of concanavalin A for 18 hrs as described by Shimizu et al. (95). The interleukin 2 was collected and

precipitated by ammonium sulfate and subsequently dialyzed in PBS. The supernate was concentrated and passed through a G-200 column. The fractions containing the IL 2 were collected and concentrated. These samples were then sterile filtered. The activity of the preparation was tested by using an IL 2 dependent cell line by Dr. Shimizu.

2.11 Preparation of Newborn Spleen Cells

Newborn mice 1 to 3 days old were sacrificed; their spleens were removed using sterile techniques. The spleens were passed through a fine wire mesh screen to get a single cell suspension. The cells were washed once with PBS and then cultured in EHAA media at a concentration of 10 x 10^6 cells/ml. Normally 2 x 10^6 viable cells are obtained per spleen.

To generate suppressor cells used for the prevention of GVH disease, the newborn cells were cultured 1 day prior to the addition of the donor cells. This mixture of cells was allowed to incubate an additional day. These cells were harvested, counted and injected into sublethally irradiated host mice.

2.12 Preparation of Alpha Fetoprotein

Pregnant mice between (10 to 14 days) were cervically dislocated and surgically opened. The amniotic sac was punctured using a needle; the amniotic fluid was then aspirated into a collection flask. The amniotic fluid was passed through an affinity column of rabbit anti-mouse AFP antibody generously provided and established by Dr. A. Kimura. The AFP was eluted off the column using glycine-HCl buffer pH 3.5. The AFP was subsequently dialyzed in PBS three times in 1000 x volume. The AFP was then tested in a primary MLR to insure that the collected substance had suppressor activity in it.

RESULTS

3.1 Inability of Allogeneic Cells to Induce GVHD in Normal Adult F_1 Mice

Adult male and female (B10 x B10.BR)F_1 mice were injected either intravenously or intraperitoneally with forty million B10.BR splenocytes. Each set of mice was observed for a period of 1 month, during which time no signs of ill health were apparent. This experiment was repeated on a new set of mice, except this time the experiment was terminated on day 5 in order to examine the spleens of these animals. The mice which were injected intraperitoneally showed no signs of illness; the spleens of these animals looked normal and possessed a normal number of leukocytes (60×10^6 cells/spleen). In contrast, the mice which were injected intravenously exhibited splenomegaly and had twice the number of leukocytes, 120×10^6 cells/spleen. Despite this enlarged spleen, the animals looked and acted like normal mice.

The spleens of these two sets of animals were prepared into single cell suspensions and forty thousand cells were dispensed into each well of a microtiter tray. The cells were then tested with a panel consisting of irradiated spleen cells from five different mouse strains as is done in a typical primed lymphocyte typing test. The cells from mice which were injected i.p. did not significantly respond towards any of the stimulating cells (Table 2, column 1). In contrast, those cells obtained from the i.v. injected mice looked larger and appeared activated. These cells did respond towards the panel of stimulating cells (Table 2, column 2); however, it was without any specific pattern.

3.2 Generation of GVHD in Adult F_1 Mice Immunosuppressed Through Irradiation

A. Study of the survival rates of mice lethally or sublethally irradiated

To obtain conditions in host animals permitting development of GVHD, it was necessary to immunosuppress the host through irradiation. A dose of irradiation was desired so that the animal would not die of infection due to the associated leukopenia, yet be compromised enough so that the donor cells would be opposed with the least possible resistance.

Table 2.
The effect of the route of injection in order to generate primed lymphocytes in the B10.BR anti-(B10 x B10.BR)F_1 reaction.

Stimulator Strain	H-2 Genetics K	A	J	E	D	^{3}H-TdR Incorporation CPM±SD (a) Mice injected by: (b) intraperitoneal	intravenous
none	-	-	-	-	-	173± 66	8570± 107
B10.BR	k	k	k	k	k	622±298	7551±1402
B10	b	b	b	b	b	525± 4	10875± 341
(B10xB10.BR)	b	b	b	b	b		
F_1	k	k	k	k	k	679±232	8046± 6797
B10.T(6R)	q	q	q	q	d	711±711	8651± 75
DBA/2	d	d	d	d	d	813± 95	9965± 835

a) PLTs were harvested at 48 hrs following a 12 hr pulse with 1 µCi of ^{3}H-TdR.
b) Primed cells were obtained on day 5 from sublethally irradiated (B10 x B10.BR)F_1 mice which were reconstituted with B10.BR splenocytes by either an intraperitoneal or intravenous route.

Figure 4 shows the survival pattern of groups of 4 mice which were irradiated with either 475, 650, 875 or 1100 rads. A dose of 475 or 650 rads produced no observable detrimental effect up to 75 days post irradiation when the experiment was terminated. Higher doses of radiation used (875 or 1100) caused death in these animals by day 14. Thus, lethal irradiation induces quick mortality while sublethal irradiation has no obvious detrimental effect on the mice.

B. Sublethally irradiated hosts provide an environment for GVHD

To test the effect of irradiation on the host in order to generate primed lymphocytes, (B10 x B10.BR)F_1 host animals were divided into two groups with one group receiving 650R while the other group did not receive any irradiation. The host animals were injected i.v. with 40 x 10^6 B10.BR splenocytes. Five days later these animals were sacrificed, their spleens were removed, and the cells tested for alloreactivity in MLR. The results of one experiment are reported in Table 3. Cells from animals which were not irradiated produced cells which did not respond specifically towards any particular strain of mouse. However, cells from those mice which were sublethally irradiated showed patterns of reactivity suggesting specific reactivity against the

Figure 4. Effect of irradiation upon mouse survival. This figure represents the survival curves of groups of mice composed of 4 individuals, in which each group received a different dose of irradiation: 475, 650, 875 and 1100 rads. Mortality was scored on the day the mouse died.

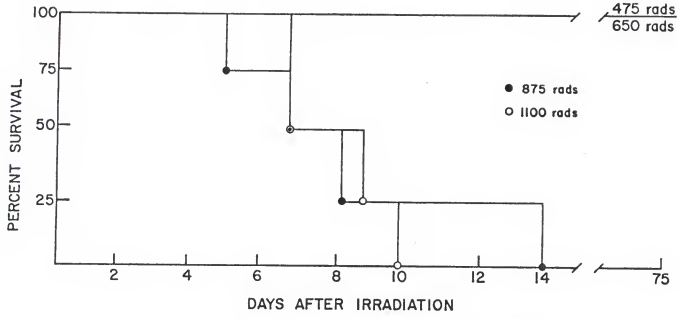

100
75
50
25
PERCENT SURVIVAL
475 rads
650 rads
● 875 rads
○ 1100 rads
2
4
6
8
10
12
14
75
DAYS AFTER IRRADIATION

Table 3.
The effect of radiation on the host in order to generate specific primed lymphocytes in the B10.BR anti-(B10 x B10.BR)F_1 reaction.

Stimulator Strain	H-2 Genetics K	A	J	E	D	^{3}H-TdR Incorporation CPM±SD (a) Condition of Host: (b) Not Irradiated	Irradiated
none	-	-	-	-	-	8570± 107	1947± 133
B10.BR	k	k	k	k	k	7551±1402	1429± 252
B10	b	b	b	b	b	10875± 341	42062±1809
(B10xB10.BR)	b	b	b	b	b		
F_1	k	k	k	k	k	8046± 697	44871± 402
B10.T(6R)	q	q	q	q	d	8651± 725	6146± 292
DBA/2	d	d	d	d	d	9965± 835	10168±2227

a) PLTs were harvested at 48 hrs following a 12 hr pulse with 1 μCi of ^{3}H-TdR.
b) Primed cells were obtained on day 5 from either sublethally irradiated or non irradiated (B10 x B10.BR)F_1 hosts which were reconstituted with B10.BR splenocytes injected i.v.

antigens which were found on the host. Thus, by immunocompromising the host it is possible to generate a GVHR.

C. Histology of sublethally irradiated (650 rads) mice

Various tissues from animals which had been previously irradiated 10 to 15 days earlier were removed, prepared for thin sectioning and then examined for abnormalities. Liver, kidney and small intestines appeared normal. Lymphoid tissue such as thymus and spleen were atrophic. The spleen contained 0.05×10^6 cells and the histology of the spleen revealed necrosis, but did show signs of regeneration with the presence of megakaryocytes.

D. General pathology of GVHD in MHC disparate strains

Sublethally irradiated animals reconstituted with 40×10^6 allogeneic or semi-allogeneic splenocytes developed acute GVHD. As early as five days postgrafting, mice started to deteriorate physiologically: The mice became lethargic, developed hunched postures, presented a wasting appearance and developed diarrhea. Often immediately before death the mice felt hypothermic and were shivering. In semi-allogeneic combinations such as (B10 x B10.BR)F_1 mice reconstituted

with B10.BR cells, mice began to die by day 7 and by day 14 to 15 all the mice had expired. In allogeneic combinations such as the B6 anti-BALB/c reaction an acclerated course of the disease was seen, and by days 5 to 6 all the mice had died.

A comparison of the survival rates due to different histocompatibility loci is presented on Figure 5. An entire H-2 disparate GVH reaction [B6 anti-BALB/c] resulted in a very short lifespan. An I region mismatch [B10.AQR anti-(B10.T(6R) x B10.AQR)F_1] produced a slightly delayed mortality, while a K/D disparate GVHD [B10.MBR anti-(A.TL x B10.MBR)F_1] resulted in about half the mice surviving the first 20 days.

E. The effect of host's age upon generation of GVHD

Another study was undertaken to determine whether age of the host had any influence on the rate of GVH mortality. In one experiment an entire H-2 combination was used: B10.BR anti-B10.WB. The age of the hosts ranged from 22 days (mice are normally weaned on day 21) to 70 days old (young adult). As can be seen in Figure 6, the majority of the mice died between days 4 to 8 and by day 10 all the mice had succumbed to GVHD. Thus, lethal GVHD developed similarly in mice

Figure 5. Lethal GVHD across major histocompatibility loci. Host BALB/c, (B10.T(6R) x B10.AQR)F_1 and (A.TL x B10.AQR)F_1 mice were sublethally irradiated with 650 rads, then reconstituted with 40 x 10^6 allogeneic B6, B10.AQR, or B10.MBR splenocytes, respectively, via the tail vein. Mortality was scored on the day the mouse died.

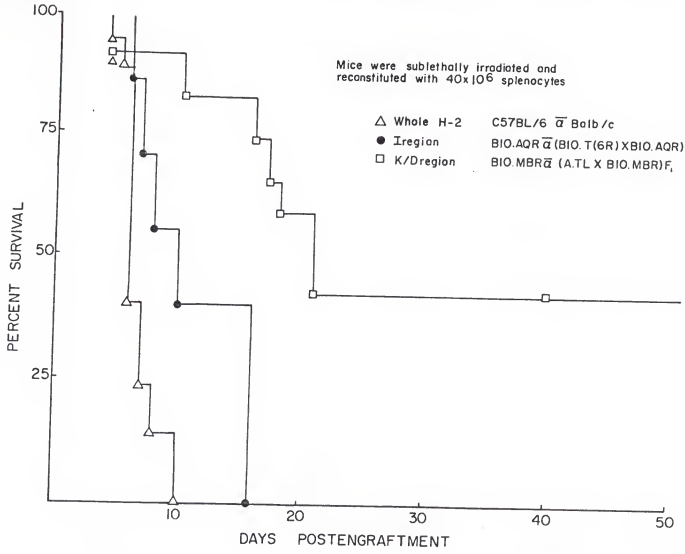
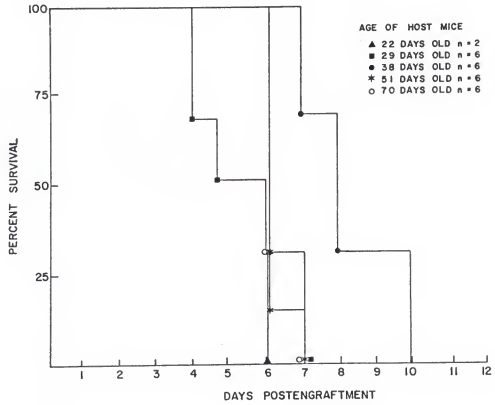

100
75
50
25
PERCENT SURVIVAL
10
20
30
40
50
DAYS POSTENGRAFTMENT
Mice were sublethally irradiated and reconstituted with 40x10^6 splenocytes
△ Whole H-2
● I region
□ K/D region
C57BL/6 α Balb/c
BIO.AQR α (BIO.T(6R) X BIO.AQR) F1
BIO.MBR α (A.TL X BIO.MBR) F1

Figure 6. The lack of a correlation of the host's age to develop lethal GVHD in a major histocompatibility mismatch: B10.BR anti-B10.WB. B10.WB mice of varying ages 22 days old to 70 days old were sublethally irradiated and reconstituted with 40 x 10^6 allogeneic B10.BR splenocytes via the tail vein. Mortality was scored on the day the mouse died.


AGE OF HOST MICE
▲ 22 DAYS OLD n = 2
■ 29 DAYS OLD n = 6
● 38 DAYS OLD n = 6
* 51 DAYS OLD n = 6
○ 70 DAYS OLD n = 6
PERCENT SURVIVAL
100
75
50
25
1
2
3
4
5
6
7
8
9
10
11
12
DAYS POSTENGRAFTMENT

ranging in age from 22 to 70 days old. Experiments performed in this study thus used mice which were this age.

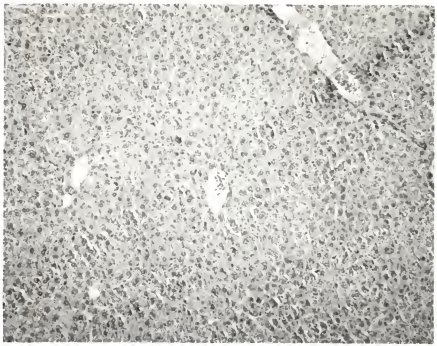
F. Histopathological examination of whole H-2 disparate GVHD revealed marked effects

The pathology described here is similar to that previously reported by Rappaport et al. (96). The livers of these GVH affected animals often changed from a normal red color to a pale white coloration. This condition seemed to frequently occur before any wasting syndrome presented itself. These livers showed signs of perivascular cuffing, dilation of the veins, massive necrosis with no signs of regeneration. There was marked evidence of leukocytic infiltration in the parenchyma as well as along the central veins (Figures 7 to 10). The yield of leukocytes from the GVH liver varied in different experiments but usually 20 x 10^3 to 70 x 10^4 cells/liver could be recovered. In contrast, normal livers failed to yield any significant amount of leukocytes.

Similarly, examination of the small intestine revealed drastic changes: the villi were dilated with columnar metaplasia. Exfoliation of the villi was also noted to be higher than normal. Leukocytes were devoid in the villi (Figures 11 and 12). Destruction and necrosis was obvious, and leukocytic and plasma cell invasion of the basement

Figure 7. Mouse liver from either a normal animal or from a sublethally irradiated animal on day 10. Both livers exhibit no drastic changes morphologically. The liver is uniformly packed with hepatocytes. Central veins are seen at lower left and center. A bile duct is seen in the upper right.

Figure 8. Normal mouse liver, a higher magnification. The previous section was examined under higher magnification. The artery is situated in the center. Notice the uniformity of the cells. Staining the cells with hematoxylin and eosin reveals the hepatocytes have an eosinophilic cytoplasm with well defined cell membranes.



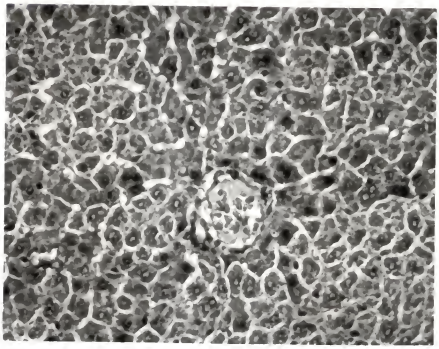
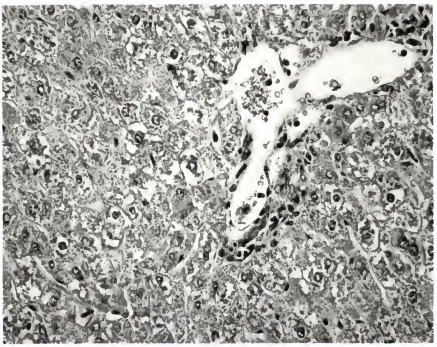


Figure 9. The liver of a mouse undergoing acute GVHD. This liver was obtained from a (B10 x B10.BR)F_1 mouse undergoing acute GVHD induced by 40 x 10^6 B10.BR splenocytes at day 10 of the reaction. Perivascular cuffing exists along the central vein. Leukocytes can be seen along the vein as well as infiltrating the parenchyma. The normal architecture of the liver appears to be disturbed with numerous vacoules found in the parenchyma.

Figure 10. A higher magnification the previous liver. Leukocytes have infiltrated along the bile duct and have invaded the parenchyma. Notice the loss of normal cellular distribution. The hepatocytes show coagulative necrosis, the cytoplasm of the cells has been disrupted with only nuclear remnants left.



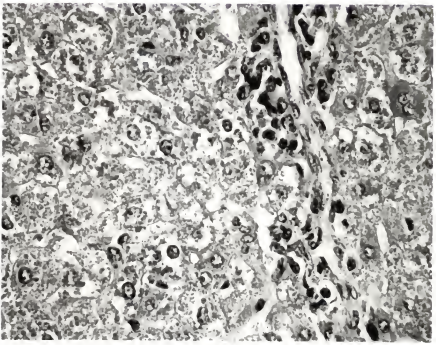
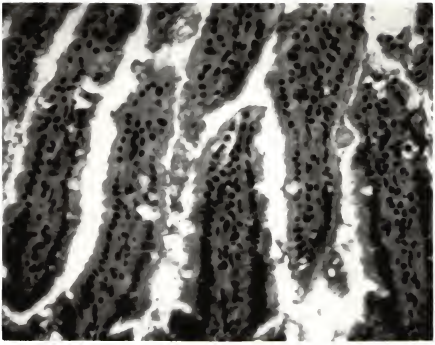
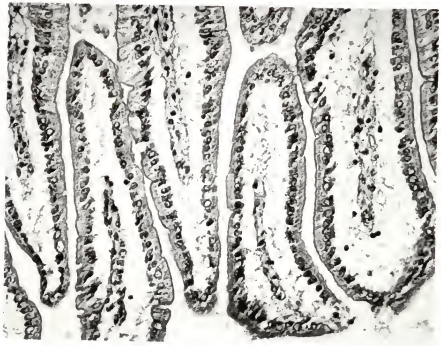


Figure 11 The intestines of a normal mouse. The intestines of a normal mouse are similar to those found in a sublethally irradiated mouse at day 10, although there is a slight decrease of (10 to 20%) leukocytes in the villi. The intestine here shows the villi are intact and have leukocytes along the lacteals.

Figure 12. The intestines of a mouse undergoing acute GVHD. A sublethally irradiated (B10 x B10.BR)F_1 mouse was injected with 40 x 10^6 B10.BR splenocytes and was examined on day 10 of the reaction. Epithelial cells are still along the periphery of each villus. Necrosis is observed in the epithelium. The lacteals are remarkedly devoid of leukocytes (90 to 95%).





membranes was pronounced. Several attempts were made to extract leukocytes from these tissues, unfortunately, no cells were recovered.

Histopathological examination of the whole H-2 GVH spleens on days 6, 10, and 15 days postgrafting revealed marked atrophy with disruption of the normal white pulp architecture, similiar to results found in the sublethally irradiated mice (Figures 13 to 17). However, more viable cells (5 to 15 x 10^6 cells/spleen) could be recovered from these types of spleens.

G. Pathology of I or K/D region disparate GVHD

In I region GVHD the spleen and liver appeared to have leukocytic infiltrates which progressed with time. The predominate cell type found in the spleen was the polymorphonuclear leukocyte (PMN)(70 to 90%). The intestines developed abnormalities in the second week after engraftment. The lesions which were seen in these animals were never as severe as those seen in whole H-2 disparate GVHDs. In addition, the pathology of one animal in a given series of GVHD was at times dissimilar to that seen in another animal indicating advanced stages of disease occurred in some animals but not in others. Mice died between 8 to 17 days after engraftment (Figure 5).

Figure 13. The spleen from a normal mouse. The germinal centers are slightly hypercellular, but the normal architecture is intact.

Figure 14. The normal spleen, a higher magnification. This section here displays two germinal centers.

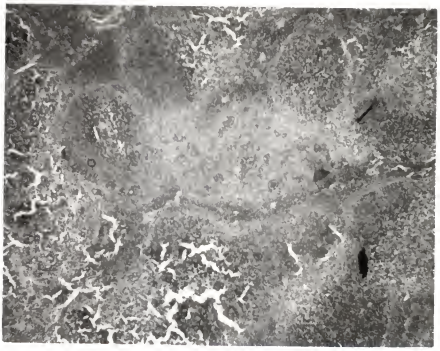
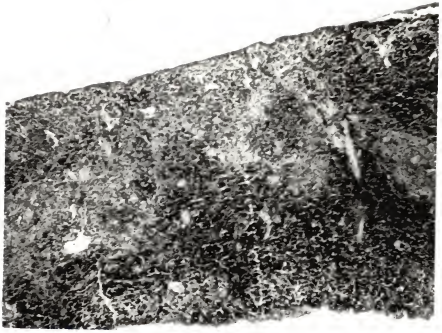


Figure 15. The spleen of an animal undergoing acute GVHD. A sublethally irradiated (B10 x B10.BR)F_1 mouse was reconstituted with 40 x 10^6 B10 splenocytes. The animal was sacrificed on day 10 of the reaction. The overall architecture of the spleen has been disrupted and appears to be identical to that produced by a sublethal dose of irradiation. The red pulp appears to be repopulated by cells.

Figure 16. A higher magnification from the previous tissue. Areas are fibrosed with collagen deposition and have numerous cells in the area. A great majority of the cells appear dead and this is confirmed when a single cell suspension is examined using trypan blue dye.



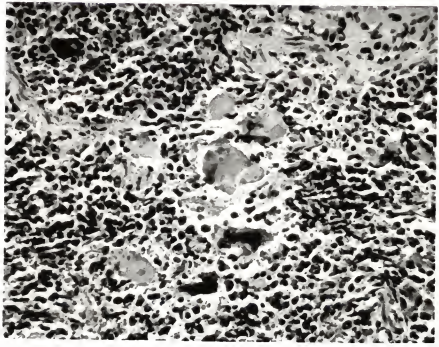
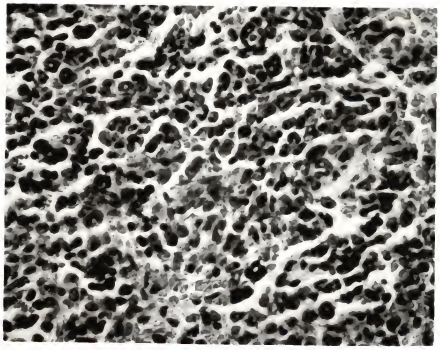
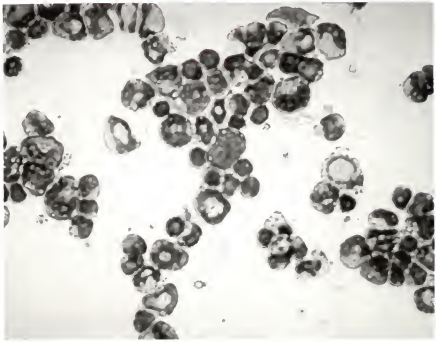


Figure 17. Further magnification of the previous spleen. The cellular infiltrate of this area appears to be mononuclear in origin. Massive amounts of necrotic cells appear to be observed in the center of the field.

Figure 18. A cytocentrifuge preparation of cells obtained from a mouse spleen undergoing acute GVHD. When the GVH spleen is passed through a wire screen and the cells are passed over ficoll gradients, to remove the dead cells, the remaining viable cells appear to show a variety of different cell types. this figure shows that about 50% of the recovered cells are PMNs. Several lymphoblasts are found along with numerous small lymphocytes.





In K/D region disparate GVHD, pathological conditions similar to those found in I region GVHD were seen; only the symptoms of the disease seemed to be delayed or absent. Death usually occurred from days 10 to 20 and some mice survived longer than four months (Figure 5). Infiltrates were found in the spleen and liver; again the predominant cell type recovered was the PMN (70 to 95%). Intestines were frequently normal; very few villi were dilated and depleted of leukocytes. However, about 60% of the animals did die in an emaciated condition.

H. Cellular composition of the host organs undergoing GVHD

Cytocentrifuge preparations of single cell suspensions of anti-H-2 GVH spleens revealed 40 to 50% of the recovered viable cells were PMNs, while 50 to 60% were lymphocyte/monocytes (Figure 18). Leukocyte preparations extracted from the livers revealed a similiar composition. Similar preparations of anti-I or anti-K/D region GVH spleens contained 70 to 90% PMNs. Culturing the leukocyte preparations overnight in tissue culture medium resulted in the majority of the PMNs dying, thereby facilitating isolation of the lymphocyte/monocyte population on ficoll-isopaque density gradients (d=1.077). Of the remaining viable cells, 30 to 35% were immunoglobulin

positive (determined by cell surface immunofluorescence); 55% stained for the Lyt 1 marker, while 20% stained for the Lyt 2 marker. Monoclonal anti-Thy 1.2 antibody plus complement killed 60% of the cells providing evidence that the majority of the cells were T cells bearing the Lyt 1 marker.

I. Recovery of viable lymphocytes from various histoincompatibility differences

The number of viable lymphocytes out of the spleen varied depending upon the genetic disparity. Table 4 summarizes these findings. The spleens of the hosts undergoing GVHD were removed on day 5 of the GVH reaction and were incubated overnight; the remaining viable lymphocytes were then enumerated the next day. In general, those combinations with the maximum genetic mismatch (entire H-2 mismatched reactions: combinations 1 to 3) yielded the most lymphocytes/spleen: 2.5 to 5.1 x 10^6. Whereas in those combinations which differed in class I molecules produced between 2.2 to 4.6 x 10^5 lymphocytes/spleen (combination 6 and 7).

Class II disparate reactions yielded varying numbers of lymphocytes. Those combinations which were directed against the I-A^k molecule yielded the most cells/spleen (combination 8 and 9) 2.8 to 4.1 x 10^6, while (B10.GD x

Table 4.
Summary of the number of recovered cells obtained from the GVH spleens on day 5 of the GVH reaction. (a)

Combination	Genetic Disparity	Cell number recovered	Number of mice used	Recovery of cells/spleen
1) B10.BR anti-(B10xB10.BR)F_1	$H\text{-}2^b$	40.0×10^6	8	5.0×10^6
2) B6 anti-BALB/c	$H\text{-}2^d$	56.5×10^6	11	5.1×10^6
3) (B10xB10.Q)F_1 anti-B10.BR	$H\text{-}2^k$	27.3×10^6	11	2.5×10^6
4) (B10.A(4R)xB10.GD)F_1 anti-B10	$K^bI\text{-}A^b$	14.6×10^6	10	1.5×10^6
5) B10.S(9R) anti-(B10.HTTxB10.A)F_1	$K^kI\text{-}A^k$	11.0×10^6	10	1.1×10^6
6) B10.MBR anti-(A.TLxB10.MBR)F_1	K^sD^d	3.2×10^6	7	4.6×10^5
7) B10.M(17R) anti-A/J	D^d	2.2×10^6	10	2.2×10^5
8) B10.S(9R) anti-(B10.HTTxB10.TL)F_1	$I\text{-}A^k$	41.0×10^6	10	4.1×10^6
9) (B10xB10.Q)F_1 anti-B10.MBR	$I\text{-}A^k$	30.7×10^6	11	2.8×10^6
10)(B10.GDxB10.MBR)F_1 anti-B10	$I\text{-}A^b$	4.8×10^6	10	4.8×10^5
11)B10.AQR anti-(B10.T(6R)xB10.AQR)F_1	$I\text{-}A^q$	4.3×10^6	6	7.2×10^5
12)B10.A(4R) anti-B10.A(2R)	$I\text{-}E^k$	2.5×10^6	4	6.3×10^5

a) Host animals were sublethally irradiated and reconstituted with 40×10^6 donor splenocytes. The animals were sacrificed on day 5. The spleens were collected and made into a single cell suspension and cultured overnight. The recovered viable cells were then counted and utilized in PLTs the next day.

B10.MBR)F_1 anti-B10 (combination 10) gave the least, 4.8 x 10^5 cells/spleen. Thus, it appears that I region GVH reactions are the most variable in terms of obtaining viable lymphocytes from the fifth day of the GVH reaction.

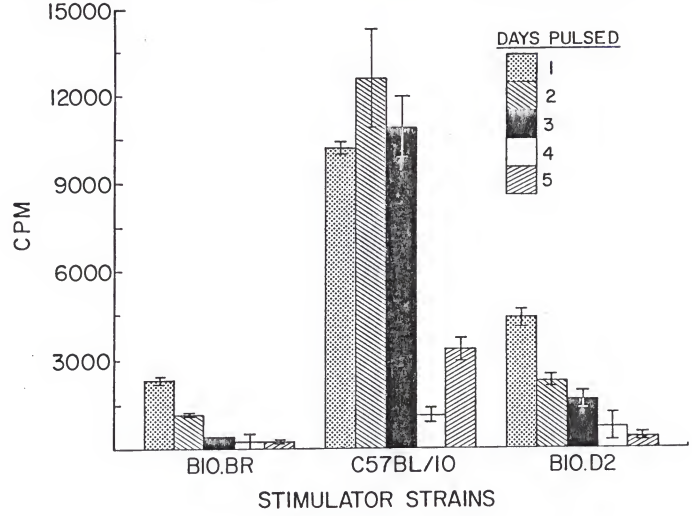
J. Functional activities of leukocytes obtained from GVH animals

The lymphocytes recovered from these GVH affected tissues were then tested in functional tests to determine what antigen(s) the recovered lymphocytes are capable of reacting. Two functional tests have been used: 1) the mixed lymphocyte reaction/primed lymphocyte typing test which measures the ability of reactive cells to proliferate against foreign histocompatibility antigens, 2) the cell mediated lympholysis test which assays the ability of primed cells to lyse ^{51}Cr labeled target cells possessing the appropriate antigens.

i. Mixed lymphocyte reaction/primed lymphocyte test. B10.BR anti-(B10 x B10.BR)F_1 reaction

The spleen cells recovered from the (B10 x B10.BR)F_1 mice undergoing GVHD induced by B10.BR cells were found capable of proliferating in a mixed lymphocyte reaction. The kinetic responses of these lymphocytes are presented in Figure 19. The activated cells reacted to B10 (H-2^b)

Figure 19. The proliferative responses of GVH primed splenocytes derived from an acute GVHR: B10.BR anti-(B10 x B10.BR)F_1. This figure demonstrates the kinetic responses of GVH primed cells obtained from the spleens of (B10 x B10.BR)F_1 mice reconstituted with B10.BR cells on day 10. The primed cells were cultured with either: B10.BR, C57BL/10 or B10.D2 irradiated stimulator cells. The various cultures were then pulsed with 1 μCi of ^{3}H-TdR for 12 hrs on days 1 to 5.


15000
12000
9000
6000
3000
CPM
DAYS PULSED
1
2
3
4
5
BIO.BR
C57BL/10
BIO.D2
STIMULATOR STRAINS

cells, expressing antigens of the F_1 host to which the B10.BR lymphocytes were sensitized. No proliferative activity was directed against the syngeneic donor cells which initiated the GVH, namely B10.BR ($H\text{-}2^k$). This indicates that the host's lymphocytes are not proliferating against the donor lymphocytes, as in the nonirradiated F_1 animals (see Table 3). Nor is reactivity seen against B10.D2, an unrelated third party haplotype ($H\text{-}2^d$). Some cross reactivity of the response of the primed cells is seen on day 1, but this is not considered significant because of the loss of activity on day 2. The optimal peak of secondary ^{3}H-TdR incorporation of either MLR or GVH primed cells is always found on day 2. Thus, the cells do not seem to be capable of reacting towards antigens which are different from those antigens which initially triggered the GVHR; and this makes this reaction appear to be similiar to the in vitro primed lymphocyte test (PLT). In addition, GVH activated lymphocytes do not react towards the T cell mitogens, concanavalin A (Con A) and phytohemagglutinin (PHA), or lipopolysaccharide (LPS), a potent murine B cell mitogen (Table 5).

(B10 x B10.BR)F_1 hybrid mice reconstituted with parental B10.BR spleen cells normally died between days 10 to 14. Cell populations recovered from the spleens and livers of mice showing signs of severe GVHD on day 8 and 9

Table 5.
Inability of GVH primed cells to respond to mitogens.

addition	^{3}H-TdR incorporation CPM±SD (a). normal cells (b)	GVH primed cells (c)	normal cells treated with anti-Thy+C' (d)	normal cells treated with anti-I-A+C' (e)
none	4668±880	1349±12	6303± -	1477± 163
Con A(f)	54043±6244	417±94	2700±595	22311±1345
LPS (g)	98977±5401	2531±81	71110±655	19737± 517

a) MLR assays were harvested at 48 hrs following a 12 hr pulse with 1 μCi ^{3}H-TdR.
b) Spleen cells from a normal healthy mouse (B10.BR) were used in this study. 5 x 10^5 cells were placed in each well of a microtiter plate.
c) (B10 x B10.BR)F_1 mice were sublethally irradiated and reconstituted with 40 x 10^6 B10.AQR splenocytes. The mice were sacrificed on day 5 after reconstitution.
d) Normal splenocytes were treated with anti-Thy 1.2 antibody and complement immediately before culturing the cells.
e) Normal splenocytes were treated with anti-I-A^k antibody and complement immediately before culturing the cells.
f) Concanavalin A dose was 5 μg/ml.
g) Lipopolysaccharide dose was 100 μg/ml.

after engraftment were compared in PLT with *in vitro* activated B10.BR anti-(B10 x B10.BR)F_1 PLT cells. As presented in Table 6, major differences exist in the response pattern of the *in vivo* and *in vitro* activated cell populations. Cells obtained from either the spleens of livers of the F_1 hybrids undergoing GVH were restimulated by cells from strains carrying the H-2^b haplotype. However, stimulation with cells from recombinant strains possessing either the H-$2D^b$ or H-$2K^b$ antigens, e.g. B10.MBR, B10.A(2R) produced little if any reactivation of proliferation, and no cross reactivity was observed on unrelated third party strains. In contrast, MLR generated PLT cells exhibited strong secondary responses to strains carrying the H-2^b haplotype as well as H-2^b haplotype derived K and D region antigens. In addition, cross reactivity on unrelated third party strains was observed such as B10.T(6R).

While a number of factors may account for the different patterns of reactivity exhibited by *in vivo* and *in vitro* primed cells, the most likely one is the protocol used herein to obtain responding PLT cell populations from organs undergoing GVH reactivity. Overnight incubations of the *in vivo* primed cells in the presence of cell debris and dying PMNs could alter markedly the reactive patterns. To determine the effect of this procedure, cell mixing

Table 6.
Comparison of PLT using B10.BR anti-(B10 x B10.BR)F_1 cells generated either in MLR or in GVH.

Stimulator strain	H-2 Genetics K	A	J	E	D	^{3}H-TdR Incorporation CPM±SD (a) In vitro primed (b)	In vivo primed (c) spleen	liver
B10.BR	k	k	k	k	k	7344± 233	1429± 252	234±127
(B10xB10.BR) F_1	b/k	b/k	b/k	b/k	b/k	92117± 3026	44871± 402	4015±984
B10	b	b	b	b	b	83972± 4	42062±1809	4467±209
B10.A(2R)	k	k	k	k	b	19769± 1744	3462±1744	278± 83
B10.MBR	b	k	k	k	q	50799±13119	2929± 11	189± 35
B10.A(4R)	k	k	b	b	b	25089± 204	2067± 264	111± 40
B10.GD	d	d	b	b	b	49169± 1409	7191±1222	319± 22
B10.A(3R)	b	b	b	k	d	68108± 1971	36910±5474	3553±458
B10.A(5R)	b	b	k	k	d	87682± 2052	34182±1667	1653±308
B10.T(6R)	q	q	q	q	d	40085± 2713	6146± 293	412±24

a) PLTs were harvested at 48 hrs following a 12 hr pulse with 1 μCi of ^{3}H-TdR.
b) Primary MLR consisted of B10.BR splenocytes incubated with irradiated (B10 x B10.BR)F_1 splenocytes for 8 days.
c) GVH reactivity was induced in sublethally irradiated (B10 x B10.BR)F_1 mice with B10.BR splenocytes. On day 8, the spleens and livers were collected, made into single cell suspensions and cultured overnight prior to use.

experiments were performed. Responding cell populations primed in the MLR of B10.BR responding against (B10 x B10.BR)F_1 were mixed with equal numbers of cells removed from the spleens of (B10 x B10.BR)F_1 mice undergoing GVH reactivity following reconstitution with B10.BR spleen cells. Following an overnight incubation, this mixed cell population was tested in PLT and its reactivity compared to the responses of the two individual cell populations. As shown in Table 7, Column 3 the mixed cell population exhibited the reactivity of the in vitro primed cell population (Column 1).

In a more direct approach to determine if differences arise due to culture artifacts, cells obtained from spleens undergoing severe GVHD separated on Ficoll-Hypaque density gradients (d=1.101) were examined directly. This procedure results in a responding purified population contaminated with less than 2% PMNs. As shown in Table 7, Column 4, the responding cell population obtained in this manner still exhibited a PLT reactivity identical to the in vivo primed cells which had been incubated overnight. Thus, the differences in the reactivity of the in vivo versus in vitro primed cells does not appear to be dependent on the handling of the cells.

K and D antigens are known to exist on every cell of the body, while I region antigens are found on only a

Table 7.
GVH primed cells fail to alter MLR primed cell responses.

Stimulator Strains	H-2 Genetics K	A	J	E	D	^{3}H-TdR Incorporation CPM±SD (a) In vitro primed cells (b)	In vivo primed cells (c)	In vivo + In vitro cells (d)	Purified in vivo cells (e)
B10.BR	k	k	k	k	k	10144±2758	1251± 12	11820±1602	1541± 387
B10	b	b	b	b	b	97862±2944	26131±1610	83250±1463	26037± 380
B10.A(3R)	b	b	b	k	d	82811±2935	23096± 373	73750±4613	35933±2444
B10.A(5R)	b	b	k	k	d	91105±1805	20935± 499	73345± 516	39068± 176
B10.M$\bar{e}$R	b	k	k	k	q	45266± 512	3858± 564	31563±3743	6270± 201
B10.A(4R)	k	k	b	b	b	28264±1404	2692± 443	26423±1596	2390± 160
B10.WB	j	j	j	j	b	33018±3188	3940± 330	29996±3005	8278±2161

a) PLTs were harvested at 48 hrs following a 12 hr pulse with 1 μCi of ^{3}H-TdR.
b) Primary MLR consisted of B10.BR splenocytes incubated with irradiated B10 splenocytes for 6 days.
c) GVH reactivity was induced in sublethally irradiated (B10 x B10.BR)F_1 mice with B10.BR splenocytes. On day 5, the spleens were collected, made into a single cell suspension and cultured overnight prior to use.
d) Equal numbers of in vitro and in vivo primed cells were cultured together overnight prior to use.
e) Single cell suspensions of GVH splenocytes were centrifuged over a Ficoll-hypaque density gradient (d=1.101). The resulting cells contained <2% PMNs. After a single wash, the resulting lymphocyte population was tested in PLT.

selected set of cells such as B cells, monocytes, skin dendritic cells and liver Kupffer cells. The possibility exists that the cells which react to the K^b and D^b antigens are filtered out before they home to the spleen or liver. To exclude this possibility, embryonic B6 mouse fibroblast monolayers were established and passaged three times. These cells possess the K^b and D^b antigens, and do not express the I-A^b antigen. In vitro primed B10.BR anti-(B10 x B10.BR)F_1 cells were then incubated for 2 hours on this monolayer and then gently rocked off the monolayer. Approximately one half of the primed cells were removed by this treatment. These cells as well as a sample of the original primed population were tested in PLT. The results shown in Table 8 show that both populations were still capable of responding to the K^b and D^b antigens found on B10.MBR and B10.GD. Although cytotoxic T cell activity was not tested before and after adsorption, it appears that this filtering mechanism does not occur in vivo because cytotoxic T cells are found in vivo in the spleen and liver and that they do respond to the K^b and D^b antigens (see below).

Treatment of the in vivo primed B10.BR anti-(B10 x B10.BR)F_1 cell populations with anti-Thy 1.2 antibody plus complement totally abolishes the PLT reactivity (Table 9). Treatment with monoclonal anti-I-A^k antibody plus

Table 8.
Absorption of MLR primed lymphocytes fails to remove proliferative responses towards K/D antigens.

Stimulator strain	H-2 Genetics K	A	J	E	D	^{3}H-TdR Incorporation CPM±SD (a) Treatment: (b) none	absorption
none	-	-	-	-	-	8236±1126	8478±2284
B10.BR	k	k	k	k	k	11043±1766	9187± 184
B6	b	b	b	b	b	88425± 648	69299±6033
B10.A(3R)	b	b	b	k	d	99479±3454	83931±1179
B10.MBR	b	k	k	k	q	15213±2087	10245±2835
B10.GD	d	d	b	b	b	52650± -	47223±6376
B10.A(4R)	k	k	b	b	b	15215± 42	15057±1344

a) PLTs were harvested at 48 hrs following a 12 hr pulse with 1 μCi of ^{3}H-TdR.

b) Primary MLR consisted of B10.BR splenocytes incubated with B10 splenocytes for 5 days. An aliqout of primed cells was incubated for 2 hrs, while the remaining cells were incubated over the B6 fibroblast monolayer for 2 hrs. After the incubation the cells were gently rocked from the plate and the lymphoblasts were recovered. The fibroblast monolayer was made by taking B6 embryoes and mincing them into single cell suspensions using a 0.5% trypsin solution. The fibroblast monolayer was passaged three times during a period of 18 days.

Table 9.
The PLT activity of GVH primed cells after treatment with various monoclonal antibodies plus complement.

stimulator cells	^{3}H-TdR incorporation CPM ±SD (a) primed cells treated with C' (b)	primed cells treated with anti-Thy + C' (c)	primed cells treated with anti-I-A + C' (d)
none	604±382	1574±544	1778±266
B10	6409±175	1490± 37	16711±880
B10.BR	649± 69	1762± 50	2252± 47
(B10xB10.BR)F_1	6361±239	1701± 33	17570±544
B10.A(3R)	5478± 59	1619± 67	14862±145
B10.A(4R)	623± 45	1152±183	3360±771
B10.A(5R)	3320±494	1128± 79	13892±951

a) GVH reactivity was induced in sublethally irradiated (B10 x B10.BR)F_1 mice with B10.BR splenocytes. Eight days later the spleens of these animals were removed and prepared into a single cell suspension and used. The cultures, consisting of 30 x 10^4 cells per well, were pulsed with 1 µCi of ^{3}H-TdR for 8 hrs on day 2 of the reaction.
b) Spleen cells recovered from the GVH reaction were treated with rabbit complement alone.
c) Spleen cells recovered from the GVH reaction were treated with anti-Thy antibody plus complement immediately before culturing.
d) Spleen cells recovered from the GVH reaction were treated with anti-I-A^k antibody plus complement immediately before culturing.

complement (kills 60% B10.BR splenocytes and 5% B10 splenocytes) a procedure which enriches for T lymphocytes by depleting the 30 to 40% contaminating B lymphocytes, increased responsiveness in those combinations already exhibiting a positive response, but did not produce reactivity against strains which showed negative responses.

C57BL6 anti-BALB/c reaction

Irradiated BALB/c mice reconstituted with B6 spleen cells normally died by day 9 to 10 after engraftment. The pathology was identical to that found in the B10.BR anti-(B10 x B10.BR)F_1 combination. Cell populations recovered from the spleens, livers and lymph nodes on day 6 were tested in PLT for their ability to mount a secondary proliferative response. As can be seen in Table 10, all three populations exhibited strong proliferative responses against cells from mice possessing the H-2^d haplotype, e.g. BALB/c and DBA/2 or from mice possessing the H-$2K^d$ and I-A^d regions, e.g. B10.GD. Little, if any significant reactivity was elicited against third party strains including those expressing H-$2D^d$ region antigens, e.g. B10.A(3R), B10.A(5R) and B10.T(6R). Thus, these alloactivated cells appear to recognize primarily determinants encoded by genes located on the left side of the H-2 complex.

Table 10.
Comparison of PLT of B6 anti-BALB/c cells generated either in MLR or in GVH.

Stimulator Strains	H-2 Genetics K	A	J	E	D	^{3}H-TdR Incorporation CPM±SD (a) In vitro primed cells (b)	In vivo primed cells (c) Spleen	Liver	Lymph nodes
B6	b	b	b	b	b	3058±1990	1203± 71	1203± 71	726± 147
BALB/c	d	d	d	d	d	70295±4777	23930± 555	23522±2619	37980±2100
DBA/2	d	d	d	d	d	97073±1100	50130±1773	49024±2128	43727±2756
B10.GD	d	d	b	b	b	55067±6394	26637±1414	20612±2598	39550±2308
B10.A(3R)	b	b	b	k	d	22429±1108	2847± 710	3216± 954	-
B10.A(5R)	b	b	k	k	d	24162±9856	1551± 909	376± 215	4068± 199
B10.T(6R)	q	q	q	q	d	22962± 953	2573± 984	1398± 469	7801± 995
B10.A(2R)	k	k	k	k	b	18534±8060	2179± 735	1273± 78	247± 66
B10.A(4R)	k	k	b	b	b	8139± 492	798± 217	1106±1044	-
SWR	q	q	q	q	q	13433± 619	1904± 85	592± 28	-
AKR	k	k	k	k	k	12294±1568	1855± 327	522± 40	-

a) PLTs were harvested at 48 hrs following a 12 hr pulse with 1 μCi of ^{3}H-TdR.
b) Primary MLR consisted of B6 splenocytes incubated with irradiated BALB/c splenocytes for 7 days.
c) GVH reactivity was induced in sublethally irradiated BALB/c mice with B6 splenocytes. On day 6, the spleens were collected, made into a single cell suspension and cultured overnight prior to use.

Secondary responses of B6 anti-BALB/c PLT primed cells primed in MLR are also included in Table 10 for comparison. These cells exhibited a quite different pattern of reactivity from GVH primed cells. Strongest responses were elicited against strains possessing the $H\text{-}2^d$ haplotype or the $H\text{-}2K^d$ and $I\text{-}A^d$ regions; however, strong responses also occurred against strains possessing the $H\text{-}2D^d$ region antigens. In addition, cross reactivity against a number of unrelated third party strains, e.g. B10.A(2R), SWR and AKR, was observed.

The B10.RIII anti-B10.A(5R) reaction

In another whole H-2 disparate GVH, B10.RIII anti-B10.A(5R), similar results were found (Table 11) like those in the two previous combinations. The MLR generated primed cells respond towards the K^b and $I\text{-}A^b$ antigens present on B10.A(5R) and B6 mice. Strong stimulation was also found on mouse cells possessing the K^b antigen B10.MBR as well as the $I\text{-}E^k$ and D^d antigens, e.g. B10.A, B10.AQR, B10.TL, B10.S(9R), A.TFR5, B10.M(17R) and B10.BR. In contrast, GVH primed cells only recognized the B10.A(5R) and B6 mouse cells. The lack of a response to the B10.MBR cells apparently eliminates stimulation due to the K^b antigen.

Table 11.
Comparison of PLT of B10.RIII anti-B10.A(5R) cells generated either in MLR or in GVH.

Stimulator Strains	H-2 Genetics K	A	J	E	D	^{3}H-TdR Incorporation CPM±SD (a) In vitro primed cells (b)	In vivo primed cells (c)
none	-	-	-	-	-	3832± 2227	489± 257
B10.RIII	r	r	r	r	r	4229± 858	151± 23
B10.A(5R)	b	b	d	d	d	85395± 1382	30006± 168
B6	b	b	b	b	b	93669± 1283	26426±4045
B10.D2	d	d	d	d	d	51594± 7504	5886±1625
B10.AQR	q	k	k	k	d	66655± 2270	2873±1453
B10.A	k	k	k	k	d	57325± 49	1704± -
B10.TL	s	k	k	k	d	52795±15596	2814±1071
B10.S(9R)	s	s	k	k	d	28673± 3896	468± 251
A.TFR5	f	f	-	k	d	32065± 8188	374± 173
B10.MBR	b	k	k	k	q	43565± 1493	2398± 155
B10.M(17R)	k	k	k	k	f	27360± 2955	1533±1327
B10.BR	k	k	k	k	k	20357± 3594	3608± 91
B10.A(4R)	k	k	b	b	b	19194± 1996	610± 1
B10.GD	d	d	b	b	b	21432± 2476	997± 250
B10.WB	j	j	j	j	b	8476± -	888± 347
B10.M	f	f	f	f	f	11401± 2331	1148± 286

a) PLTs were harvested at 48 hrs following a 12 hr pulse with 1 μCi of ^{3}H-TdR.
b) Primary MLR consisted of B10.RIII splenocytes incubated with irradiated B10.A(5R) splenocytes for 6 days.
c) GVH reactivity was induced in sublethally irradiated B10.A(5R) mice with B10.RIII splenocytes. On day 5, the spleens were collected, made into a single cell suspension and cultured overnight prior to use.

Again, the GVH primed cells display a more restricted response pattern than do primed cells established *in vitro*.

The B10.GD anti-B10.M(17R) and B10.WB anti-B10.M(17R) reactions

Two other genetic combinations with H-2 disparate regions studied were B10.GD anti-B10.M(17R) and B10.WB anti-B10.M(17R) as shown in Table 12. Both *in vitro* primed cell populations responded towards cells from mice which possessed either the K^k,$I\text{-}A^k$ or D^f molecules. In contrast, the GVH primed cells reacted only to those cells which possessed the $I\text{-}A^k$ molecule, e.g. B10.BR, B10.M(17R), B10.MBR, B10.AQR and B10.A(4R). Although minor reactivity is seen against E^k and D^f molecules, the GVH primed cells clearly did not respond to the same magnitude as the *in vitro* primed cells do.

Even though the B10.GD anti-B10.M(17R) and B10.WB anti-B10.M(17R) reactions are directed against the B10.M(17R) haplotype, these two *in vitro* reactions are not identical. The B10.GD anti-B10.M(17R) primed cells recognized some determinants which the B10.WB anti-B10.M(17R) primed cells did not, such as those found on the B10.F cells. What these determinants are is not readily distinguished from the present data. However, this antigenic determinant is readily detected by the *in vivo* primed cells giving some

Table 12.
Comparison of PLT of anti-B10.M(17R) reactive cells generated either in MLT or in GVH.

Stimulator Strains	H-2 Genetics K	A	J	E	D	^{3}H-TdR Incorporation CPM±SD (a) In vitro primed (b) B10.GD anti-B10.M(17R)	In vitro primed (b) B10.WB anti-B10.M(17R)	In vivo primed (c) B10.GD anti-B10.M(17R)	In vivo primed (c) B10.WB anti-B10.M(17R)
none	-	-	-	-	-	1594± 281	1552± 638	97± 55	579± 23
B10.GD	d	d	b	b	b	1115± 367	4062±1191	2998± 0	968± 303
B10.D2	d	d	d	d	d	2539± 199	-	1561± 194	2864± 317
B10.WB	j	j	j	j	b	-	2093± 506	-	614± 146
B10.M(17R)	k	k	k	k	f	44694± 1549	24304±1946	34989±2594	28647± 984
B10.BR	k	k	k	k	k	55622± 1332	26465± 271	39904±3731	36015± 921
B10.MBR	b	k	k	k	q	44515±12015	24192±2344	36908± 0	35239±1383
B10.AQR	q	k	k	k	d	39051± 2837	43982±2751	36945±4965	35172±3935
B10.A(4R)	k	k	b	b	b	18540±14060	34730±3325	27239±3950	21024±5081
B10.S(9R)	s	s	k	k	d	17084± 799	4483± 757	7790± 598	1620± 506
B10.M	f	f	f	f	f	34894± -	12080±1502	9843± 300	3645± 376
B10.RIII	r	r	r	r	r	-	4489± 134	-	2164± 158
B10.S	s	s	s	s	s	353± 341	-	4462± 366	-
B10.F	p	p	p	p	p	21093± 184	9035± 833	12240±1264	3509± 290
B10.T(6R)	q	q	q	q	d	9027± 904	-	5535± 306	-

a) PLTs were harvested at 48 hrs after a 12 hr pulse with 1 μCi of ^{3}H-TdR.
b) Primary MLR consisted of either B10.GD or B10.WB splenocytes incubated with irradiated B10.M(17R) splenocytes for 6 days.
c) GVH reactivity was induced in sublethally irradiated B10.M(17R) mice with either B10.GD or B10.WB splenocytes. On day 5, the spleens were collected, made into single cell suspensions and cultured overnight prior to use.

possibility that these cross reactions could either be due to cross reactivity to the I-A^p molecule which is common to the I-A^k molecule or that the I-E^p cross reacts with the I-E^k molecule. The latter explanation is favored because the B10.GD does not express the I-E molecule and is therefore capable of recognizing the entire I-E molecule as foreign, while the B10.WB animal does express the I-E molecule and does not have to respond to an entire I-E molecule as would the B10.GD cells.

The B10.M(17R) anti-B10.GD and B10.WB anti-B10.GD reactions

When another set of whole H-2 reactions is established against the B10.GD mouse [B10.M(17R) anti-B10.GD and B10.WB anti-B10.GD] a different pattern of data is seen (Table 13). The predominate <u>in vitro</u> stimulatory antigens come from the left side of the H-2 complex, i.e. K^d and I-A^d as exhibited by B10.GD and B10.D2. Reactivity is also observed on B6, B10.A(3R) and B10.A(4R) cells which possess the I-J^b and D^b antigens. In addition, cross reactivity on the I-A^b molecule is also postulated for the B6 and B10.A(3R) cells, but these responses to the antigens do not seem to be additive when compared to the B10.A(4R) response. This cross reactivity on I-A^b could be due to the shared antigens Ia8 and Ia15 which I-A^d has in common with I-A^b to which the I-A^k molecule lacks. In addition, the GVH

Table 13.
Comparison of PLT of anti-B10.GD reactive cells generated either in MLR or in GVH.

Stimulator Strains	H-2 Genetics K	A	J	E	D	^{3}H-TdR Incorporation CPM±SD (a) In vitro primed (b) B10.M(17R) anti-B10.GD	B10.WB anti-B10.GD	In vivo primed (c) B10.M(17R) anti-B10.GD	B10.WB anti-B10.GD
none	-	-	-	-	-	355± 247	2699± 19	262± 245	417±226
B10.WB	j	j	j	j	b	-	2243± 286	-	396±146
B10.M(17R)	k	k	k	k	f	1910± 83	4204± 743	241± 2	350±120
B10.GD	d	d	b	b	b	56739±1411	30192±1219	34250±4848	9604±556
B10.D2	d	d	d	d	d	67771±7652	33813± 197	38583± 921	19700±851
B10.A(3R)	b	b	b	d	d	26543±2309	9305± 193	11502± 765	1912±253
B6	b	b	b	b	b	25131± -	14670±1843	11175± -	2035± 78
B10.A(4R)	k	k	b	b	b	41841± 861	6302± 137	1278± 83	869± 47
B10.BR	k	k	k	k	k	5766±1809	4062±1102	186± 17	652±227
B10.MBR	b	k	k	k	q	4720±1587	4981± 90	239± 34	476±218
B10.AQR	q	k	k	k	d	2612±3271	6343± 689	209± 38	540± 10
B10.HTT	s	s	s	k	d	12072±2399	-	2025±1145	-
B10.TL	s	k	k	k	d	14107±1206	4762± 838	537± 141	439±199
B10.S(9R)	s	s	k	k	d	13030± 436	4309± 46	2053± 368	326± 74
B10.S	s	s	s	s	s	13043±2728	-	2811±1221	-
B10.M	f	f	f	f	f	1203± 214	4775± 687	2091± 471	1071± 52
B10.Q	q	q	q	q	q	34612±3890	-	10860± 921	-
B10.RIII	r	r	r	r	r	-	4519± 550	-	452± 1

a) PLTs were harvested at 48 hrs following a 12 hr pulse with 1 μCi of ^{3}H-TdR.
b) Primary MLR consisted of either B10.M(17R) or B10.WB splenocytes incubated with irradiated B10.WB splenocytes for 6 days.
c) GVH reactivity was induced in sublethally irradiated B10.GD mice with either B10.M(17R) or B10.WB splenocytes. On day 5, the spleens were collected, made into single cell suspensions and cultured overnight prior to use.

primed cells also appear to recognize these antigens, since GVH primed cells apparently only respond to I region antigens. Thus, this cross reactivity is likely to be due to class I antigens.

Interestingly, the in vitro B10.M(17R) anti-B10.GD reaction appears to detect a cross reaction on the K^s molecule, since B10.TL, B10.S, B10.S(9R) and B10.HTT cells induce stimulation by the primed cells. In contrast, the GVH primed cells do not appear to recognize this cross reactivity; again the response seen by the GVH primed cells is different from those of the MLR generated cells. These cross reactions to K^s which are seen by the in vitro primed B10.M(17R) anti-B10.GD cells are not seen by the in vitro primed B10.WB anti-B10.GD cells.

The I region GVH reactions

A number of mouse strain combinations exists with limited I region disparity. Two of the most extensively studied combinations are B10.A(4R) responding against B10.A(2R), two congenic lines differing genetically between the H-2 I-A and D, and B10.AQR responding against B10.T(6R), two congenic lines differing genetically throughout the I regions. These two combinations are considered to represent, respectively, an anti-I-E^k and anti-I-A^q response. The secondary MLR responses of B10.A(4R) anti-B10.A(2R) and

B10.AQR anti-(B10.T(6R) x B10.AQR)F_1 PLT obtained from 10 day GVH spleens or primary MLR are examined in Table 14.

As presented in Table 14, the PLT cells generated in MLR exhibited strong responses against the primary stimulating strains as well as strong cross reactivity on third party strains. For example, in the B10.A(4R) anti-B10.A(2R) reaction the specific antigen is I-$E\alpha^k\beta^k$, yet B10.HTT which has I-$E_\alpha{}^k{}_\beta{}^s$ is recognized by the in vitro primed cells as well as the B10.RIII cells. Likewise, a great deal of cross reactivity is seen by the B10.AQR anti-(B10.T(6R) x B10.AQR)F_1 reaction, a reaction supposedly directed at the I-A^q molecule. Mouse cells from B10, B10.S(7R), B10.M and B10.RIII mice elicit a response by these primed cells. Again as was demonstrated with the other reactions the GVH primed cells demonstrate marked specificity. In the B10.A(4R) anti-B10.A(2R) reaction the GVH primed cells only respond to the specific antigen: I-E $\alpha^k\beta^k$ found on B10.AQR, B10.A(2R) and B10.A. While in the B10.AQR anti-B10.T(6R) reaction only those cells possessing the I-A^q molecule found on B10.T(6R) and DBA/1 cause restimulation.

K/D region GVH reactions

Two strains namely B10.MBR and A.TL possess genetic differences at only K and D loci. The MLR and GVH reactivity

Table 14.
Comparison of PLT of anti-H-2I region reactive cells generated either in MLR or in GVH.

Stimulator Strain	H-2 Genetics K	A	J	E	D	^{3}H-TdR Incorporation CPM±SD (a) B10.A(4R) anti-B10.A(2R) In vitro primed (b)	B10.A(4R) anti-B10.A(2R) In vivo primed (c)	B10.AQR anti-B10.T(6R) In vitro primed (b)	B10.AQR anti-B10.T(6R) In vivo primed (c)
B10.A(4R)	k	k	b	b	b	5508± 923	1028± 99	-	-
B10.A(2R)	k	k	k	k	b	18098±1283	7279±2468	-	-
B10.A	k	k	k	k	d	19144± 623	5076± 164	-	-
B10.AQR	q	k	k	k	d	19653± 894	8910± 411	4317± 217	1151± 290
B10.BR	k	k	k	k	k	-	-	4108±1154	1018± 173
B10.T(6R)	q	q	q	q	d	-	-	44961± 479	4228± 149
DBA/1	q	q	q	q	q	-	-	37127±1407	6525±1883
B10	b	b	b	b	b	-	-	22317± 761	1896± 293
B10.HTT	s	s	s	k	d	15773± 628	1144± 21	-	-
B10.S(7R)	s	s	s	s	d	6443±1821	1226± 250	10222± 498	1794± 60
B10.M	f	f	f	f	f	5448±1411	1191± 43	9572± 908	1092± 177
B10.RIII	r	r	r	r	r	16912± 272	1595± 45	10016± 782	1078± 130

a) PLTs were harvested at 48 hrs following a 12 hr pulse with 1 μCi of ^{3}H-TdR.
b) Primary MLRs contained responding cells incubated with irradiated stimulator splenocytes for 8 days.
c) GVH reactivity was induced in sublethally irradiated B10.A(2R) or (B10.T(6R) x B10.AQR)F$_1$ mice with B10.A(4R) or B10.AQR splenocytes, respectively. On day 9, the spleens were collected, made into single cell suspensions and cultured overnight prior to use.

of these combinations have been examined. Results from the reaction of B10.MBR responding against (B10.MBR x A.TL)F_1 hybrids, which involves a haplotype mismatch at both the K and D, is presented in Table 15. PLT cells generated *in vitro* exhibited strong secondary responses against third party strains expressing K^s/D^d antigens, syngeneic with the primary stimulating antigens. No cross reactivity was observed with other haplotype products. Such specific reactivity against class I products is well documented. In contrast, it has not been possible to obtain primed cells from organs undergoing GVH in K/D disparate combinations which will proliferate against K^s or D^d region antigens in PLT, despite the fact that the mice are dying from GVHD.

Another class I mismatched reaction was explored on a gross mortality level, namely the B6 anti-$B6^{bml}$ reaction and the $B6^{bml}$ anti-B6 reaction. This combination represents a mismatch directed at a variant K^b molecule. The K^b molecule found on $B6^{bml}$ cells represents a K molecule with 2 amino acid substitutions (Wakeland, personal communication). The reactions observed proved to be one way reactions: one half of the $B6^{bml}$ mice reconstituted with B6 cells died within the first 15 days of the reaction, while none of the B6 mice reconstituted with the $B6^{bml}$ cells succumbed to death. Thus, a variant molecule is

Table 15.
Comparison of PLT of B10.MBR anti-(A.TL x B10.MBR)F_1 cells generated either in MLR or in GVH.

Stimulator Strains	H-2 Genetics K	A	J	E	D	^{3}H-TdR Incorporation CPM±SD (a) In vitro primed cells (b)	In vivo primed cells (c)
B10.MBR	b	k	k	k	q	1921± 37	397± 57
(A.TLxB10.MBR) F_1	b/s	k	k	k	q/d	21241±2715	264± 15
B10.TL	s	k	k	k	d	28455±2710	203± 40
B10.S(7R)	s	s	s	s	d	26055± 121	-
B10.S(9R)	s	s	k	k	d	20570±1066	257± 7
B10.HTT	s	s	s	k	d	24706± 610	258± 35
B10.T(6R)	q	q	q	q	d	21645± 227	240± 57
B10.D2	d	d	d	d	d	27624±1587	184± 4
B10	b	b	b	b	b	4597± 264	135±132
B10.BR	k	k	k	k	k	4661± 184	365±100
B10.GD	d	d	b	b	b	5653± 938	243± 25

a) PLTs were harvested at 48 hrs following a 12 pulse with 1 μCi of ^{3}H-TdR.
b) Primary MLR consisted of B10.MBR splenocytes incubated with irradiated (A.TL x B10.MBR)F_1 splenocytes for 7 days.
c) GVH reactivity was induced in sublethally irradiated (A.TL x B10.MBR)F_1 mice with B10.MBR splenocytes. On day 6, the spleens were collected, made into a single cell suspension and cultured overnight prior to use.

sufficient to induce lethal GVHD, but for some unknown reason the individual possessing the variant molecule is not capable of producing a lethal reaction in those animals which possess the parent molecule.

Minor histocompatibility GVH reactions

In the in vitro MLR system there exists another antigenic system which is known to cause strong T lymphocyte proliferation similar to I region antigens. This system has been termed Mls. The Mls antigen system was originally described by Festenstein (97, 98, 99) by using the primary MLR on H-2 identical mice. The original reaction came from the combination BALB/c anti-DBA/2. Later by using the F_1 tests and PLT assays other various mouse strains have been given Mls designations.

BALB/c (Mls^b) cells which express the silent Mls allele are able to respond against cells which have the Mls^a antigen such as is found on DBA/2 cells. Indeed, in vitro primed cells appear to be able to proliferate towards mouse strains which apparently are Mls^a positive, i.e. DBA/2, Dl.C, AKR and possibly SEA (Table 16). In addition, positive reactions are also seen on Mls^d positive cells: CBA/J and RF. Likewise, Festenstein has given the mice DBA/2, Dl.C, NZB, SM, DBA/1 and AKR the designation Mls^a, while CBA/J and RF are Mls^d (101) and these two

Table 16.
Comparison of PLT of BALB/c anti-DBA/2 cells generated in either MLR or in GVH.

Stimulator strain	H-2	Mls	^{3}H-TdR Incorporation CPM±SD (a)	
			In vitro primed (b)	In vivo primed (c)
none	-	-	309± 8	1819±684
BALB/c	d	b	10615± 496	1610±180
SEC	d	b	42867±3975	1713± 25
SEA	d	a	90114±2165	1905±373
DBA/2	d	a	80777±2568	5390±231
D1.C	d	a	92882±3137	3438±484
B10.BR	k	b	-	1735±412
CBA/Ca	k	b	-	2072±911
RF	k	d	83890±2890	-
CBA/J	k	d	-	3534± 54
C3H/He	k	c	34850±1975	1934± 23

a) PLTs were harvested at 48 hrs following a 12 hr pulse with 1 μCi of ^{3}H-TdR.
b) Primary MLR consisted of BALB/c splenocytes incubated with irradiated DBA/2 splenocytes for 6 days.
c) GVH reactivity was induced in sublethally irradiated DBA/2 mice with BALB/c splenocytes. On day 5, the spleens were collected, made into a single cell suspension and cultured overnight prior to use.

designations cross react strongly and are possibly the same antigen (100, 101). However, since SEA induced strong secondary proliferation, its Mls phenotype is assumed to be either $Mls^{a \text{ or } d}$. Similar findings are exhibited by B10.D2 anti-DBA/2 primed cells (Table 18 column 3).

As is the case found in I region GVH, Mls disparate GVHs produced very few lymphoblasts in the spleen but an increased amount of PMNs frequently interferes with PLT assays. However, GVH primed cells do appear to proliferate when Mls^{a} or Mls^{d} positive cells, e.g. DBA/2, D1.C, SEA and (RF or CBA/J) are cultured with the primed cells (Table 16 Column 2).

Another Mls disparate GVH was established using CBA/Ca (Mls^{b}) cells reacting against AKR (Mls^{a}) cells. The results are shown in Table 17. In this experiment positive results are seen by proliferation responses against AKR, DBA/2 and SEA cells. Again this reaction shows weak responses directed against the Mls^{a} determinant in contrast to the in vitro reactions where strong responses are demonstrated.

Whereas the in vivo primed cells failed to yield large numbers of primed cells towards minor histocompatibility antigens such as Mls, it was possible to examine and define Mls antigens carried by various mouse strains by utilizing in vitro MLR primed cells. From the

Table 17.
Comparison of PLT responses of CBA/Ca anti-AKR generated in either MLR or in GVH.

Stimulator cells	H-2	Mls	^{3}H-TdR incorporation CPM ±SD (a) In vitro primed (b)	In vivo primed (c)
none	-	-	4919± 152	1317± 199
CBA/Ca	k	b	4137± 311	1381± 227
B10.BR	k	b	3315± 365	1990± 75
AKR	k	a	89997±2524	6811± 72
C3H/He	k	c	21202± 53	1385± 349
BALB/c	d	b	44178±7094	3535± 116
B10.D2	d	b	20362± 113	4488± 226
SEC	d	b	23793± 714	4832± 503
DBA/2	d	a	97441±2568	10155±1014
SEA	d	a	67067±1496	7502±1318

a) PLTs were harvested at 48 hrs following a 12 hr pulse with 1 μCi of ^{3}H-TdR.
b) Primary MLR consisted of CBA/Ca splenocytes incubated with irradiated AKR splenocytes. PLT cells were collected on day 5.
c) Sublethally irradiated AKR mice received CBA/Ca splenocytes. At 9 days after reconstitution, the spleens were collected, prepared into a single cell suspension and cultured overnight prior to use.

Table 18.
PLT responses of anti-DBA/2 primed cells generated in MLR.

Stimulator Strains	H-2	Mls	Responder Cells CPM ± SD (a) (B10.D2xAKR)F_1	(BALB/cxAKR)F_1	B10.D2	SEA	NZB
none	-	-	449± 323	469± 287	3818± 992	786±317	973± 86
BALB/c	d	b	5537± 489	-	14488± 802	-	-
SEC	d	b	7434± 270	-	-	-	-
B10.D2	d	b	3851± 615	-	5155±3691	-	-
NZB	d	a	17775± 394	-	-	383± 0	1534± 117
SEA	d	a?	11494±2065	-	-	1516±406	733± 301
DBA/2	d	a	12620±1024	143366±4045	59988± 513	9688±478	6730±1880
D1.C	d	a	23988± 252	175522±1749	71759±2503	-	-
CBA/J	k	d	-	639± 94	-	-	1719± 443
AKR	k	a	4781± 394	657± 86	56355±9569	3443±616	2025± 170
RF	k	d	-	-	31823±1983	-	
C58	k	a?	19392± 62	-	-	-	-
C57BR	k	b?	3638±1112	-	-	-	-
CE	k	a?	-	-	49530± 226	-	-
C3H/He	k	c	-	-	18105± 962	1986±1044	-

a) PLTs were harvested at 48 hrs following a 12 hr pulse with 1 μCi of ^{3}H-TdR. Primary MLR consisted of responder splenocytes incubated with irradiated DBA/2 splenocytes. PLT cells were collected on days 6 to 9.

reactions described here, BALB/c anti-DBA/2 (Table 16) and CBA/Ca anti-AKR (Table 17), PLT analysis have identified DBA/2, Dl.C, RF, AKR, and SEA as being Mls similar.

When further experimentation was performed on the genetics of the Mls, it was noted that this system is not as simple as it was inititially described. For example, when F_1 hybrid mice were constructed [(B10.D2 x AKR)F_1 and (BALB/c x AKR)F_1] so that the Mls^a antigen was blocked out by the AKR background, these F_1 mice demonstrated strong proliferation against DBA/2 cells. This would indicate that another antigen is capable of causing in vitro proliferation other than Mls and H-2. Further studies have shown that SEA and NZB probably lack this antigen or antigens because they also are capable of recognizing DBA/2 and Dl.C cells in PLT assays (Table 18 columns 4 and 5).

Additional experiments have shown that different strains of mice may recognize minor histocompatibility antigens in the context of the H-2 antigens. For example, in Table 19 the B10.PL anti-PL and B10.SM anti-SM combinations produce good primary reactions only in one direction and the primed cells respond in PLT assays. From PLT assays, it appears that the $Mls^{a/d}$ antigens are responsible for some of the stimulation which is observed because the primed cells respond towards cells from DBA/2, SEA, Dl.C, AKR and NZB.

Table 19.
Summary of minor histocompatibility in vitro assays in inbred H-2 mice.

Reaction:	H-2	Primary Reaction (a)	PLT (b)
B10.PL anti-PL	u	+	+
PL anti-B10.PL	u	-	-
B10.SM anti-SM	v	+	+
SM anti-B10.SM	v	-	-
B10.S anti-SJL	s	-	-
SJL anti-B10.S	s	+	+
C3H.NB anti-B10.F	p	-	-
B10.F anti-C3H.NB	p	-	-
SWR anti-B10.Q	q	+	-
SWR anti-DBA/1	q	+	-
B10.Q anti-SWR	q	+	-
B10.Q anti-DBA/1	q	+	-
(B10.BRxSWR)F_1 anti-SWR	q	+	-
BALB/c anti-DBA/2	d	+	+
B10.D2 anti-DBA/2	d	+	+
SEA anti-DBA/2	d	+	+
SEC anti-DBA/2	d	+	+
NZB anti-DBA/2	d	+	+
AKR anti-CBA/J	k	+	-
AKR anti-C58	k	+	-
CBA/J anti-C58	k	+	-
CBA/J anti-AKR	k	+	-
C58 anti-CBA/J	k	+	-
C58 anti-AKR	k	+	-
AKR anti-RF	k	-	-
CBA/Ca anti-AKR	k	+	+

a) The ability to generate a positive primary MLR is demonstrated by either + or -.
b) The ability of the primed lymphocytes to respond and give a specific PLT is demonstrated by either + or -.

Using mice carrying the H-2^q and H-2^k haplotype strange reactions are found. In the H-2^q system, SWR reacts strongly to DBA/1 and B10.Q, B10.Q reacts strongly to SWR and DBA/1, while (B10.BR x SWR)F_1 reacts strongly to SWR. Despite these strong primary reactions with large numbers of blast cells, no specific PLT reactivity has been obtained to date. These primed cells recognize self as strongly as they recognize the original stimulating cells. Likewise in the H-2^k system certain combinations yielded good primary MLRs, yet no specific PLT activity was obtained. The possibility exists that the primary reaction was still continuing when the primed cells were taken and this would explain why no specific pattern was detected. However, this explanation does not appear likely, since these primed cells could be extracted 2 weeks after the culture was established and the cells have had sufficient time to revert back to small lymphocytes. Again these cells failed to show any specific proliferation.

Another combination yeilding interpretable results was SJL reacting against B10.S. Here the primed cells reacted against B10.S, CE and C58, whereas AKR, DBA/2, SM, PL and CBA/J cells did not induce any proliferation by these primed cells. Thus, it appeared there may be another minor histocompatibilty reaction which did not appear to involve the Mls system.

ii. Cell mediated lympholysis assays

Results of the PLT tests in entire H-2 mismatched combinations raised the question of whether any reactivity against K/D antigens in vivo could be found. For this reason cells recovered from organs undergoing GVH in each combination were tested for cytolytic activity in CML against targets expressing K/D (or I) antigens syngeneic with the primary stimulating cells.

B10.BR anti-(B10 x B10.BR)F_1 reaction

Results presented in Table 20 using the B10.BR anti-(B10 x B10.BR)F_1 system indicate that killer cells can be recovered from the spleen on day 5 of the GVH reaction. MLR generated cells lysed cells which express either the K^b or D^b, e.g. B10.MBR and B10.A(2R). Cells recovered from the GVH spleen were capable of similar patterns of killing as exhibited by the MLR generated killer cells. If the cells were cultured overnight to get rid of the PMNs, no killing activity was exhibited. However, if these cells were cultured overnight with EL-4 derived interleukin 2, killing activity was retained, but restricted to the cells which possess the K^b antigen.

Table 20.
CML reactivity of primed B10.BR anti-(B10 x B10.BR)F_1 cells generated either in MLR or in GVH.

Target cells	H-2 Genetics K	A	J	E	D	% Specific Release (a) MLR generated (b) 50:1	25:1	GVH generated (c) Day 0 6:1	Day 1 37:1	18:1
B10.BR	k	k	k	k	k	11	5	-12	-4	-1
B10	b	b	b	b	b	90	50	15	22	10
B10.A(5R)	b	b	k	k	d	49	27	12	18	9
B10.MBR	b	k	k	k	q	46	32	9	17	10
B10.A(2R)	k	k	k	k	b	50	-	15	-8	-10
B10.RIII	r	r	r	r	r	26	-	-	8	-1

a) This test was performed in a 6 hr assay.
b) Primary MLR consisted of B10.BR splenocytes incubated with irradiated (B10 x B10.BR)F_1 splenocytes for 5 days. The effector to target ratios used were: 50:1 and 25:1.
c) GVH reactivity was induced in sublethally irradiated (B10 x B10.BR)F_1 mice with B10.BR splenocytes. On day 5, the spleens were collected and made into a single cell suspension and assayed the same day (day 0) or were cultured overnight in interleukin 2 overnight and used the next day (day 1).

C57BL/6 anti-BALB/c reaction

The results presented in Table 21 compare the CML reactivity of primed B6 ($H\text{-}2^b$) cells generated against BALB/c ($H\text{-}2^d$) cells derived from either a mixed lymphocyte reaction or from animals undergoing GVHD from day 5. Both sets of GVH primed cells lysed those cells which express the host's K^d or D^d antigens as found on BALB/c, B10.GD, B10.A, B10.T(6R) and B10.S(9R), but did not lyse the B6 cells or unrelated third party strains such as B10.A(2R) lysed.

The killing activity of GVH primed cells appeared to be identical, whether these cells resided in the spleen or the liver (Table 21 column 3 and 4). The surface phenotypes of both GVH populations are Lyt 1+ (60 to 70%), Lyt 2+ (20%) and Ig+ (20 to 30%). In addition, both sets of cells proliferate only towards the $I\text{-}A^d$ antigen (data shown in Table 10). Thus, both sets of GVH primed cells apparently are identical, with the only difference being that they were isolated from two different organs.

Prior treatment of the splenic cytotoxic cells with monoclonal anti-Thy 1.2 (HO-13-4) plus complement abolishes the ability of these cells to lyse ^{51}Cr labeled BALB/c and B10.GD targets (Table 21 column 5). Addition of anti-Lyt 2 antibody (53-6.7) but not anti-Lyt 1 antibody (53-13.3) to CML culture also inhibits the lysis of targets similar to

Table 21.
CML reactivity of primed B6 anti-BALB/c cells generated either in MLR or in GVH.

Target cells	H-2 Genetics K	A	J	E	D	% Specific Release (a) MLR generated (b) 37:1	18:1	GVH generated (c) Liver 37:1	Spleen 37:1	Treated Spleen 37:1
B6	b	b	b	b	b	3	-4	-2	4	-
B10.A(2R)	k	k	k	k	b	-1	-3	9	7	-
BALB/c	d	d	d	d	d	23	17	25	32	2
B10.GD	d	d	b	b	b	17	13	16	20	-4
B10.A	k	k	k	k	d	18	12	17	20	-
B10.T(6R)	q	q	q	q	d	20	11	20	25	-
B10.S(9R)	s	s	k	k	d	15	11	17	22	-

a) This test was performed in a 6 hr assay.
b) Primary MLR consisted of B6 splenocytes incubated with irradiated BALB/c splenocytes for 5 days. The MLR primed cells were assayed at either 37:1 or 18:1 effector to target ratios.
c) GVH reactivity was induced in sublethally irradiated BALB/c mice with B6 splenocytes. On day 6, the spleens and livers were collected, made into a single cell suspension and cultured overnight in interleukin 2 prior to use. The GVH primed cells were assayed at 37:1 effector to target ratios. The GVH primed spleen cells were treated with anti-Thy antibody plus complement immediately before being assayed.

that reported previously (102,103). In addition, cytotoxic cells derived from GVH animals must be maintained with exogenous interleukin 2; otherwise killing activity is lost. From these findings it appears that cytotoxic T cells are indeed present in either the spleens or the livers of animals undergoing acute GVHD across an entire H-2 haplotype.

B10.MBR anti-(A.TL x B10.MBR)F_1 reaction

In a set of experiments involving incompatibility at the H-2K plus D regions weak, but transient, CTLs developed in host organs undergoing severe GVH disease (Table 22). (A.TL x B10.MBR)F_1 hosts were reconstituted with B10.MBR cells. Cells from afflicted spleens of these mice were subsequently examined for CML reactivity from days 6 through 19. As can be seen in Table 22, weak but transient CML activity could be detected against target cells expressing the H-2D^d gene product, e.g., B10.D2 and B10.TL during the early stages of the reaction (between days 5 to 8). However, by day 19 when severe GVH disease was obvious, no cytotoxic activity could be recovered from the affected spleens. In contrast, CTLs generated in MLR in the same combination exhibited strong CML reactivity against B10.TL, B10.S and B10.D2 target cells expressing H-2K^s and/or H-2D^d gene products.

Table 22.
CML reactivity of primed B10.MBR anti-(A.TL x B10.MBR)F_1 cells generated either in MLR or in GVH.

Target cells	H-2 Genetics K	A	J	E	D	% Specific Rlease (a) MLR generated (b) 40:1	20:1	GVH generated (c) Day 6 40:1	Day 22 70:1
B10.MBR	b	k	k	k	q	-4	-3	-18	-12
A.TL	s	k	k	k	d	26	18	12	1
B10.S	s	s	s	s	s	7	8	-10	-3
B10.D2	d	d	d	d	d	16	8	12	-5
B10.BR	k	k	k	k	k	-1	-3	-	-12
B6	b	b	b	b	b	0	3	5	-

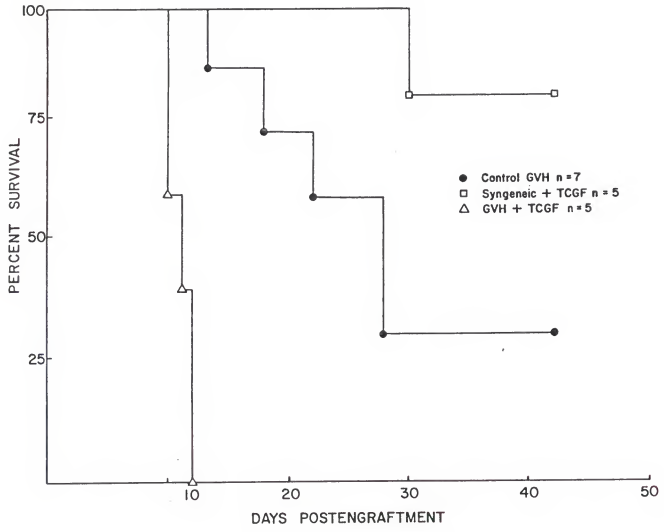
a) This test was performed in a 6 hr assay.
b) Primary MLR consisted of B10.MBR splenocytes incubated with irradiated (A.TL x B10.MBR)F_1 splenocytes for 5 days. The MLR primed cells were assayed at either 40:1 or 20:1 effector to target ratios.
c) GVH reactivity was induced in sublethally irradiated (A.TL x B10.MBR)F_1 mice with B10.MBR splenocytes. On days 6 and 22, the spleens were collected, made into single cell suspensions and cultured overnight in interleukin 2 and assayed the next day. The day 6 GVH primed cells were assayed at a 40:1 effector to target ratio, while the day 22 GVH primed cells were assayed at a 70:1 effector to target ratio.

Histologically, the picture of GVHD in this combination was similar, although not as severe as in the entire H-2 difference. The number of recovered viable cells from the spleen was markedly lower than in the entire H-2 difference with only 5×10^5 viable cells recovered per spleen as compared to 5×10^6 recovered in the entire H-2 GVH. Cytocentrifuge preparations of these recovered cells showed a marked predominance of PMNs (90 to 95%). From these findings it can not be ruled out that the cytotoxic cells have migrated out of the spleen and into the body and have contributed to the tissue destruction seen histologically.

Ability of purified interleukin 2 to speed up mortality in K/D disparate GVHD

In order to test the hypothesis whether increased cytotoxic activity could be induced in the K/D disparate GVH, two groups of (A.TL x B10.MBR)F_1 mice were reconstituted with B10.MBR cells, with one group receiving 10 units of EL-4 derived IL 2 every second day up to day 7, while the other group did not receive any IL 2. The purified IL 2 had no mitogenic activity on resting splenocytes as determined by tritiated thymidine incorporation. In addition, this IL 2 had no deleterious effect on sublethally irradiated mice reconstituted with syngeneic splenocytes up to 30 days postengraftment (Figure 20). The pattern of K/D

Figure 20. The effect of purified IL 2 given to mice undergoing K/D GVHD: B10.MBR anti-(A.TL x B10.MBR)F_1.(A.TL x B10.MBR)F_1 mice were sublethally irradiated with 650 rads and reconstituted with 40 x 10^6 B10.MBR splenocytes via the tail vein. The experimental mice and syngeneic reconstituted mice recieved 10 units of purified IL 2 on days 1,3 and 5 via the tail vein. The control GVH mice received an equal aliqout of tissue culture media which contained no IL 2. Mortality was scored on the day the mouse died.


100
75
50
25
PERCENT SURVIVAL
10
20
30
40
50
DAYS POSTENGRAFTMENT
Control GVH n = 7
Syngeneic + TCGF n = 5
GVH + TCGF n = 5

disparate GVHD again exhibited the survival pattern as seen in Figure 5. However, those mice which received both the K/D allogeneic splenocytes and the IL 2 developed mortality rates which were comparable to an entire H-2 disparate GVHD. Thus, this exogenous IL 2 supplemented to a weak GVH significantly accelerates the disease process.

The presence of CTL activity was examined in those mice developing acute GVH with the help of the exogenous IL 2. The results of such a study are seen in Table 23. This experiment reveals that cells obtained from the spleens from either the normal GVH or from the IL 2 supplemented GVH possessed identical equivalents of CTL activity. Similar results were seen in a combination involving only a D disparate GVHR, namely B10.M(17R) anti-A/J (Table 24).

Histologically, the IL 2 supplemented GVHR had a disease which appeared to have progressed further than the normal K/D disparate GVH. Here the IL 2 supplemented GVH mice had increased amounts of liver necrosis along with increased numbers of infiltrating leukocytes in the parenchyma.

B10.AQR anti-(B10.T(6R) x B10.AQR)F_1 reaction

GVH induced by I region incompatibility did not result in readily detected cytotoxic responses. Figure 5 shows that such I region disparate mismatches resulted in the host animals dying with GVHD somewhat slower than hosts with

Table 23.
CML reactivity of GVH primed B10.MBR anti-(A.TL x B10.MBR)F_1 supplemented in vivo with or without interleukin 2.(a)

						% Specific Release (b)					
	H-2 Genetics					Control GVH			GVH + in vivo IL 2		
Targets	K	A	J	E	D	40:1	20:1	10:1	40:1	20:1	10:1
B10.MBR	b	k	k	k	q	-18	-14	-9	-16	-17	-11
B10.TL	s	k	k	k	d	12	9	4	9	4	0
B10.S	s	s	s	s	s	-10	-4	-5	-9	-10	-12
B10.D2	d	d	d	d	d	12	14	7	15	10	3
B10	b	b	b	b	b	5	-2	-5	-4	-4	-5

a) GVH reactivity was induced in sublethally irradiated (A.TL x B10.MBR)F_1 mice with B10.MBR splenocytes. One half of the mice received 10 units of interleukin 2 i.v. on days 1,3 and 5. On day 6, the spleens were collected, made into single cell suspensions and cultured overnight in interleukin 2 overnight. The cells were assayed at either: 40:1, 20:1or 10:1 effector to target ratios.
b) This test was performed in a 6 hr assay.

Table 24.
CML reactivity of GVH primed B10.M(17R) anti-A/J cells supplemented in vivo with or without interleukin 2. (a)

Target cells	H-2 Genetics K	A	J	E	D	% Specific Release (b) Control GVH 17:1	8:1	GVH + in vivo IL2 17:1	8:1
B10.M (17R)	k	k	k	d	f	-7	-3	-4	-4
A/J	k	k	k	d	d	20	14	19	17

a) GVH reactivity was induced in sublethally irradiated A/J mice with B10.M(17R) splenocytes. One half of the mice received 10 units of interleukin 2 i.v. on days 1,3 and 5. On day 6, the spleens were collected, made into single cell suspensions and cultured in interleukin 2 overnight. The cells were assayed at either 17:1 or 8:1 effector to target ratios.
b) This test was performed in a 6 hr assay.

entire H-2 disparate GVH, but faster than the K/D disparate GVH. Yet, no cytotoxic activity against the host was detected in GVH on day 5 to 14. Representative data from day 6 is presented in Table 25. Again, as in the case of the K/D difference, the predominant cell type found in the spleen was the PMN (90 to 95%).

To rule out the possibility that cytotoxic cells were present, but in low and undetectable numbers for functional tests, 650R irradiated (B10.T(6R) x B10.AQR)F_1 mice were again reconstituted with B10.AQR cells. These mice were sacrificed on day 6, their splenocytes removed and expanded for 2 weeks with IL 2. These cells were then tested in a CML assay and the results are shown in Table 26. The results show that CTL cells were present in these animals and these CTLs lysed B10.T(6R) B cell blasts (LPS stimulated) but not B10.T(6R) T cell blasts (Con A stimulated) (experiment 1).

Besides a cytotoxic response directed towards cells expressing the $I\text{-}A^q$ molecule found on B10.T(6R) B cells, cytotoxic responses were also demonstrated against several distinct target cells, e.g. B10.AQR, B10.A, B10.A(2R) and B10.A(4R). Since B10.MBR cells were not lysed, the $I\text{-}A^k$ molecule is probably not the target antigen. Further support that the antigen(s) seen by these GVH primed cells is not directed at the $I\text{-}A^k$ molecule was provided when anti-$I\text{-}A^k$ monoclonal antibody (10.2.16) did not block killing

Table 25.
CML reactivity of primed B10.AQR anti-(B10.T(6R) x B10.AQR)F_1 cells generated in either MLR or in GVH.

	Specific Release (a)	
Target cells	In vitro primed (b)	In vivo primed (c)
B10.AQR Con A blasts	-10	16
B10.AQR LPS blasts	-11	-5
B10.T(6R) Con A blasts	-11	-4
B10.T(6R) LPS blasts	-28	-6

a) This test was performed in a 6 hr assay.
b) The primary MLR consisted of B10.AQR splenocytes incubated with irradiated (B10.T(6R) x B10.AQR)F_1 splenocytes for 5 days. The MLR primed cells were assayed at a 15:1 effector to target ratio.
c) GVH reactivity was induced in sublethally irradiated (B10.T(6R) x B10.AQR)F_1 mice with B10.AQR splenocytes. On day 5 the spleens were collected, made into a single cell suspension and assayed the same day. The GVH primed cells were used at a 15:1 effector to target ratio.

Table 26.
CML reactivity of GVH primed B10.AQR anti-(B10.T(6R) x B10.AQR)F_1 cells expanded in vitro with interleukin 2.(a)

Target cells	H-2 Genetics K	A	J	E	D	% Specific Release (b) Experiment 1 (c) 20:1	10:1	5:1	Experiment 2 (d) 10:1	5:1
B10.T(6R) Con A	q	q	q	q	d	-2	1	-2	-	-
B10.T(6R) LPS						12	11	7	-	-
B10.AQR LPS	q	k	k	k	d	57	55	52	32	18
B10.A LPS	k	k	k	k	d	-	-	-	60	79
B10.A(2R) LPS	k	k	k	k	b	-	-	-	52	45
B10.A(4R) LPS	k	k	b	b	b	-	-	-	51	48
B10.A(5R) LPS	b	b	k	k	d	-	-	-	-13	-4
B10.MBR LPS	b	k	k	k	q	-	-	-	-10	-11

a) GVH reactivity was induced in sublethally irradiated (B10.T(6R) x B10.AQR)F_1 mice with B10.AQR splenocytes. On day 5, the spleens were collected, made into a single cell suspension and fed interleukin 2 every second day.
b) This test was performed in a 6 hr assay.
c) This experiment was done on the tenth day in culture.
d) This experiment was done on the nineteenth day in culture.

of the B10.AQR LPS blasts by these CTLs (Table 27). Yet, this antibody is indeed capable of blocking PLT responses directed against the I-A^k molecule using MLR primed B10.T(6R) anti-B10.AQR cells.

Further studies using these cytotoxic cells revealed that these cells were incapable of killing B6, B10.F, B10.M, B10.D2 and B10.S LPS blasts (Table 28). These cells were tested on the 42^{nd} day after they were removed from the animals and showed that their lytic activity had changed. These cells now no longer had the capacity to kill B10.AQR blasts as strongly as before but were now only able to kill B10.BR blasts, both LPS and Con A blasts.

It thus appears that this cell line originally contained at least 3 populations of cytotoxic cells: one directed against the B10.T(6R) LPS blasts, presumably I-A^q, the second directed against B10.AQR LPS blasts and the third population which was heteroclitic in nature which responded to B10.BR LPS and Con A blasts.

It should be pointed out that the addition of 20 units of purified IL 2 to the I region GVH reaction did not alter the rate of mortality in this system (Figure 21). These data presented in this combination demonstrate that a host versus graft (HVG) reaction is possible even though the host was irradiated with a near lethal dose of radiation (650R). The histopathological lesions of HVG are identical to those

Figure 21. The effect of purified IL 2 given to mice undergoing I region GVHD: B10.AQR anti-(B10.T(6R) x B10.AQR)F_1. (B10.T(6R) x B10.AQR)F_1 mice were sublethally irradiated with 650 rads and reconstituted with 40 x 10^6 B10.AQR splenocytes via the tail vein. The experimental mice received 20 units of IL 2 on days 1,3 and 5 via the tail vein. Mortality was scored on the same day the mouse died.

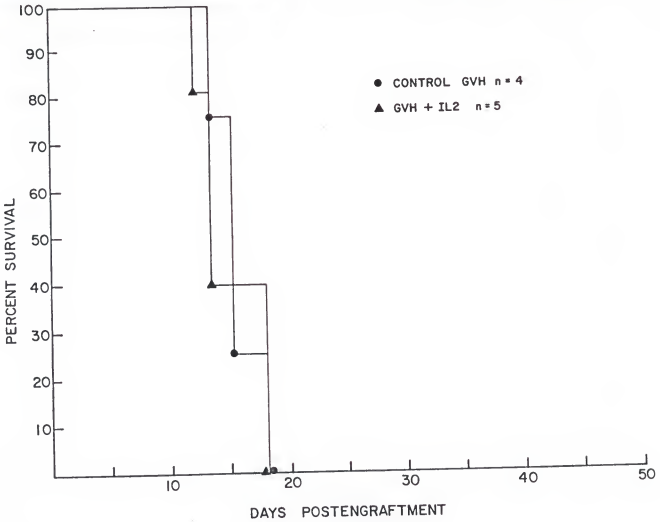

100
90
80
70
60
50
40
30
20
10
PERCENT SURVIVAL
CONTROL GVH n = 4
GVH + IL2 n = 5
10
20
30
40
50
DAYS POSTENGRAFTMENT

Table 27.
The inability of monoclonal antibodies to block killing of B10.AQR LPS blasts by GVH primed B10.AQR anti-(B10.T(6R) x B10.AQR)F_1 cells expanded in vitro with interleukin 2. (a)

condition	% Specific Release (b)	% Inhibition
no addition	57	-
10.2.16 1:20 (c)	65	0
10.2.16 1:40	59	0
B 312 1:20 (d)	52	9
B 312 1:40	54	5
14-4-4 1:20 (e)	64	0
14-4-4 1:40	59	0
anti-Lyt 1 1:10	71	0
anti-Lyt 2 1:10	50	9
anti-Thy 1:10	71	0

a) GVH reactivity was induced in sublethally irradiated (B10.T(6R) x B10.AQR)F_1 mice with B10.AQR splenocytes. On day 6, the spleens were collected, made into a single cell suspension and cultured in interleukin 2 every second day. The experiment was done on day 29. The effector to target ratio was 10:1.
b) This test was performed in a 4 hr assay.
c) Anti-I-A^k antibody (10.2.16) with a final dilution of 1:20 was used to block lysis.
d) Anti-K^d antibody (B 312) with a final dilution of 1:20 was used to block lysis.
e) Anti-I-E^k antibody (14-4-4) with a final dilution ofd 1:20 was used to block lysis.

Table 28.
CML reactivity of GVH primed B10.AQR anti-(B10.T(6R) x B10.AQR)F_1 cells expanded with interleukin 2. (a)

Target cells	H-2 Genetics K	A	J	E	D	% Specific Release (b) Experiment 1 (c) 12:1	6:1	3:1	Experiment 2 (d) 16:1	8:1	4:1
B10.AQR LPS	q	k	k	k	d	9	4	2	7	7	2
B10.AQR Con A						-	-	-	-1	0	-1
B10.BR LPS	k	k	k	k	k	22	19	15	-	-	-
B10.BR Con A						-	-	-	22	17	13
B10.TL Con A	s	k	k	k	d	-	-	-	6	7	6
B6 LPS	b	b	b	b	b	-4	1	-4	-	-	-
B10.F LPS	p	p	p	p	p	-5	-9	-8	-	-	-
B10.M LPS	f	f	f	f	f	-10	-12	-7	-	-	-
B10.D2 LPS	d	d	d	d	d	-12	-2	-3	-	-	-
B10.S LPS	s	s	s	s	s	-13	-29	-6	-	-	-

a) GVH reactivity was induced in sublethally irradiated (B10.T(6R) x B10.AQR)F_1 mice with B10.AQR splenocytes. On day 5, the spleens were collected, made into a single cell suspension and fed interleukin 2 every second day.
b) This test was performed in a 6 hr assay.
c) This experiment was done on the forty second day in culture.
d) This experiment was done on the fifty fifth day in culture.

produced in GVH (104); thus, it could be argued that in this combination both types of reactions are occurring and that the CTL activity is undetectable because the ^{51}Cr assay does not detect the activity of these few cells.

It is interesting to note that the rate of mortality in B10.T(6R) anti-(B10.T(6R) x B10.AQR)F_1 combination is identical to that B10.AQR anti-(B10.T(6R) x B10.AQR)F_1 reaction (Figure 22).

Other I region mismatches

A second combination involving an I-A^k mismatch (B10 x B10.Q)F_1 anti-B10.MBR has also been examined. As can be seen in Figure 23 the mortality rate in this combination proved comparable to an entire H-2 mismatch [(B10 x B10.Q)F_1 anti-B10.BR]. When the spleen cells from these I region GVH mice were grown in vitro in the presence of IL 2 for 1 week, CTL activity was demonstrable against both the donor haplotype cells B6 and B10.Q, as well as the host cells B10.MBR (Table 29).

Another I region mismatch namely (B10.MBR x B10.GD)F_1 reacting against B10, an I-A^b disparate reaction has also been studied. Figure 24 illustrates the mortality kinetics of such a reaction. (B10.A(4R) x B10.GD)F_1 anti-B10 provides both K^b and I-A^b differences and demonstrates a strong lethal GVH reaction. In contrast, the I-A^b

Figure 22. Lethal GVHD in sublethally irradiated (B10.T(6R) x B10.AQR)F_1. (B10.T(6R) x B10.AQR)F_1 mice were sublethally irradiated with 650 rads and reconstituted with 40 x 10^6 allogeneic splenocytes via the tail vein. B10.T(6R) cells induced a GVHR directed at ther $I\text{-}A^k$ $I\text{-}E^k$ molecules, while the B10.AQR cells induced a GVHR directed at the $I\text{-}A^q$ molecule. Mortality was scored on the day the mouse died.

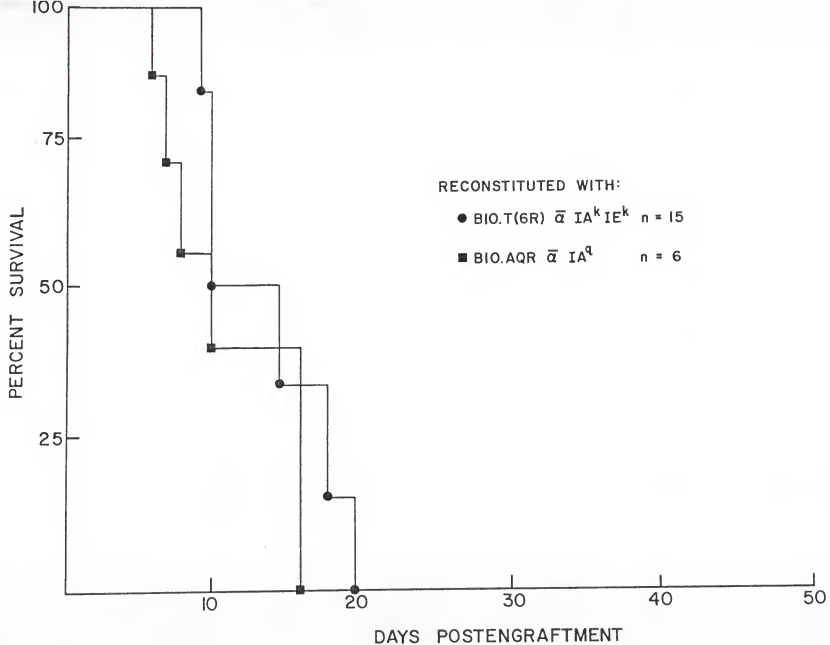

100
75
50
25
PERCENT SURVIVAL
10
20
30
40
50
DAYS POSTENGRAFTMENT
RECONSTITUTED WITH:
● BIO.T(6R) $\bar{\alpha}$ IA^k IE^k n = 15
■ BIO.AQR $\bar{\alpha}$ IA^q n = 6

Figure 23. Lethal GVHD across K^k $I\text{-}A^k$ or $I\text{-}A^k$ region differences. Forty million (B10 x B10.Q)F_1 splenocytes were injected i.v. via the tail vein into either sublethally irradiated B10.BR mice, a K^k $I\text{-}A^k$ disparate combination, or into B10.MBR mice, an $I\text{-}A^k$ disparate combination. Mortality was scored on the day the mouse died.

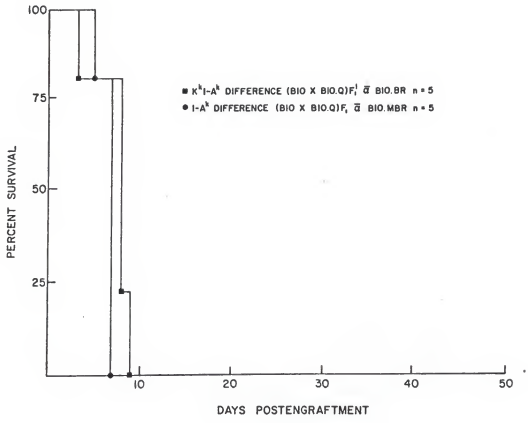
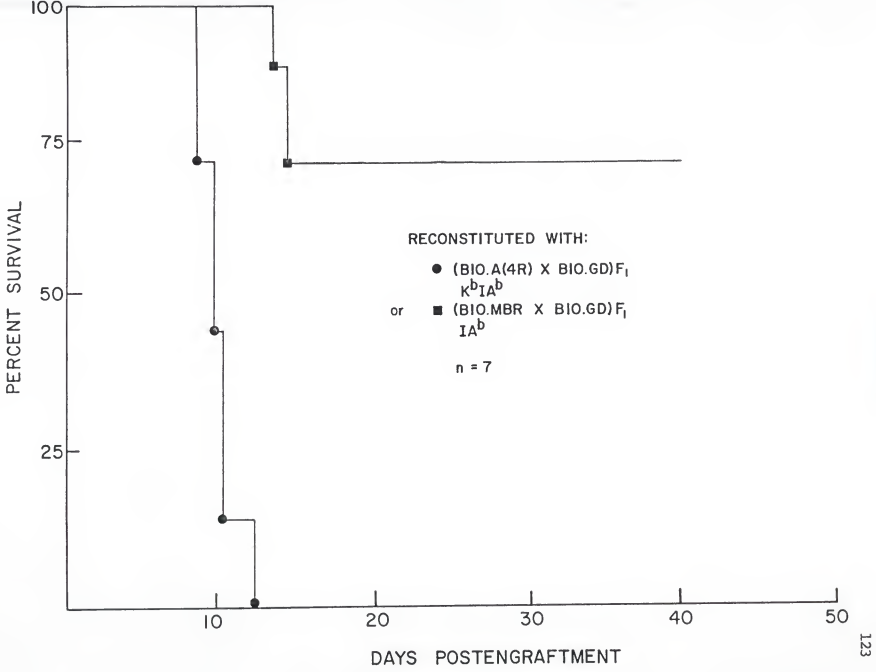

100
75
50
25
PERCENT SURVIVAL
■ K^kI-A^k DIFFERENCE (BIO X BIO.Q)F_1^1 ⊼ BIO.BR n = 5
● I-A^k DIFFERENCE (BIO X BIO.Q)F_1 ⊼ BIO.MBR n = 5
10
20
30
40
50
DAYS POSTENGRAFTMENT

Table 29.
CML reactivity of GVH primed (B10 x B10.Q)F_1 anti-B10.MBR cells expanded with interleukin 2. (a)

Target cells	H-2 Genetics K	A	J	E	D	% Specific Release (b) 14:1 (c)
B6 LPS blasts	b	b	b	b	b	13
B10.Q LPS blasts	q	q	q	q	q	18
B10.MBR LPS blasts	b	k	k	k	q	22
B10.MBR Con A blasts						17
B10.S(9R) LPS blasts	s	s	s	s	s	-4

a) B10.MBR mice were sublethally irradiated and reconstituted with (B10 x B10.Q)F_1 splenocytes. On day 5 these mice were sacrificed and their spleens were removed. A single cell suspension of cells was then fed purified interleukin 2 every second day for 1 week.
b) This test was performed in a 6 hr assay.
c) The effector to target ratio was 14:1.

Figure 24. Lethal GVHD in sublethally irradiated C57Bl/10. C57BL/10 mice were sublethally irradiated with 650 rads and reconstituted with 40 x 10^6 allogeneic splenocytes via the tail vein. (B10.A(4R) x B10.GD)F_1 splenocytes induced a GVHR directed at the K^b $I\text{-}A^b$ molecules, while (B10.MBR x B10.GD)F_1 splenocytes induced a GVHR directed at the $I\text{-}A^b$ molecule. Mortality was scored the day the mouse died.


100
75
50
25
PERCENT SURVIVAL
RECONSTITUTED WITH:
● (BIO.A(4R) X BIO.GD)F1
K^bIA^b
or ■ (BIO.MBR X BIO.GD)F1
IA^b
n = 7
10
20
30
40
50
DAYS POSTENGRAFTMENT

differences, (B10.MBR x B10.GD)F_1 anti-B6, only showed a mild reaction. When the splenocytes were recovered and expanded *in vitro* for 1 week with purified IL 2, a weak cytotoxic response was directed against the B6 LPS targets, while little if any lysis was seen against either of the donor cell lines (Table 30). It would appear that in this reaction only a graft versus host reaction was occurring and that the weak mortality which was demonstrated *in vivo* is the result of a weak cytolytic response of T killer cells.

In contrast, in GVH reactions, such as B10.HTT anti-(B10.TL x B10.HTT)F_1, no mortality was observed (Figure 25). When GVH primed cells from these animals were grown in the presence of IL 2 and tested for CTL activity, no detectable lympholysis could be observed (Table 31).

Minor histocompatibility antigens

The presence of *in vivo* CTLs was next explored in those mice which produced low but detectable PLT cells towards minor histocompatibility antigens. Minor histocompatibility disparate GVH reactions were established using the DBA/2 mouse as the host. Survival times of DBA/2 mice reconstituted with BALB/c, B10.D2 and BALB/c^{dm2} are presented in Figure 26. DBA/2 hosts grafted with BALB/c splenocytes showed only 8% mortality over the first 45 days, while the DBA/2 mice receiving the B10.D2 cells showed a

Table 30.
CML reactivity of GVH primed (B10.MBR x B10.GD)F_1 anti-B6 cells expanded with interleukin 2. (a).

Target cells	% Specific Release (b) 16:1	8:1	4:1
B6 Con A blasts	4	1	1
B6 LPS blasts	9	6	5
B10.MBR Con A blasts	-1	-3	-1
B10.MBR LPS blasts	2	4	0
B10.GD Con A blasts	3	2	4
B10.GD LPS blasts	0	1	0

a) GVH reactivity was induced in sublethally irradiated B10.MBR mice with (B10 x B10.Q)F_1 splenocytes. On day 5, the spleens were collected, made into a single cell suspension and cultured in interleukin 2 every second day. The cells were tested on day 14.
b) This test was performed in a 6 hr assay. The effector to target ratios used were:16:1,8:1 and 4:1.

Figure 25. Lethal GVHD in K^k $I\text{-}A^k$ or in $I\text{-}A^k$ mismatches. Forty million B10.HTT splenocytes were injected i.v. via the tail vein into sublethally irradiated (B10.A x B10.HTT)F_1 mice, a K^k $I\text{-}A^k$ disparate combination, or into (B10.TL x B10.HTT)F_1 mice, an $I\text{-}A^k$ disparate combination. Mortality was scored on the day the mouse died.

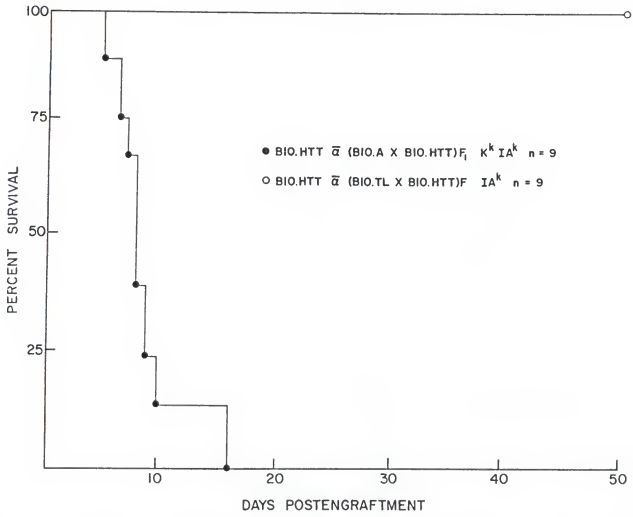
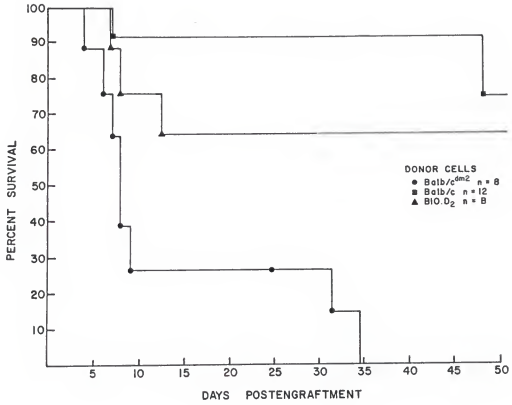

100
75
50
25
PERCENT SURVIVAL
10
20
30
40
50
DAYS POSTENGRAFTMENT
● BIO.HTT ᾱ (BIO.A X BIO.HTT)F1 K^k IA^k n = 9
○ BIO.HTT ᾱ (BIO.TL X BIO.HTT)F IA^k n = 9

Table 31.
Lack of CML reactivity of GVH primed B10.HTT anti-(B10.TL x B10.HTT)F_1 cells expanded with interleukin 2. (a).

Target cells	H-2 Genetics K	A	J	E	D	% Specific Release (b) 40:1	20:1	10:1
B10.HTT Con A	s	s	k	k	d	-11	-5	-9
B10.HTT LPS						-9	-10	-4
B10.TL Con A	s	k	k	k	d	-7	-5	-3
B10.TL LPS						-14	-12	-12
B10.S(9R) Con A	s	s	k	k	d	-4	-6	-5
B10.S(9R) LPS						-1	-3	-3
DBA/1 LPS	q	q	q	q	q	-9	-3	-3
SEA LPS	d	d	d	d	d	-4	-6	-4

a) GVH reactivity was induced in sublethally irradiated (B10.TL x B10.HTT)F_1 mice with B10.HTT splenocytes. On day 5, the spleens were collected, made into a single cell suspension and fed interleukin 2 every second day for 1 week.
b) This test was performed in a 6 hr assay.

Figure 26. Effect of minor histocompatibility antigens on survival in DBA/2 mice. Forty million donor cells were injected i.v. via the tail vein into sublethally irradiated DBA/2 mice. B10.D2 and BALB/c splenocytes reacting against DBA/2 antigens represent Mls disparate reactions plus other minor histocompatibility antigens. BALB/c^{dm2} splenocytes represent a Mls disparate reaction as well as an anti-L^d response, since the BALB/c^{dm2} mice lack a L^d molecule.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40
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20
10
PERCENT SURVIVAL
5
10
15
20
25
30
35
40
45
50
DAYS POSTENGRAFTMENT
DONOR CELLS
● Balb/c^{dm2} n = 8
■ Balb/c n = 12
▲ B10.D$_2$ n = 8

mortality rate of 37% by day 13. However, even in this latter set some mice survived several months. In contrast, a different picture was observed when the BALB/c mutant mouse strain, BALB/c^{dm2}, was used as the source of the donor cells. The BALB/c^{dm2} mouse does not express the H-2L molecule, so any reactivity which occurs is directed against both the minor histocompatibility antigens of DBA/2 and the H-2L molecule. This reaction resulted in a complete mortality by day 34 with the majority of the mice dying by day 9.

When CTL activity was assayed in the BALB/c anti-DBA/2 combination on day 5 of the GVHR and after culturing the cells in IL 2, no significant killing was observed (Table 32). If the GVH primed cells were tested in CML the same day they were removed from the host animals so that the contaminating PMNs (which comprised 85% of the total viable cells) were present no killing was observed even in a 48 hr assay (Table 33). Thus, PMNs and non-MHC primed cells were not capable of killing ^{51}Cr labeled targets.

The survival curve of sublethally irradiated AKR mice reconstituted with CBA/CaH cells is presented in Figure 27. Three out of 5 mice died within the first 17 days. Those animals not showing acute GVHD survived several months without any indication of disease. This survival was similar to that seen previously in the K/D disparate GVHD.

Table 32.
CML reactivity of primed BALB/c anti-DBA/2 cells generated in GVH

Target cells	H-2	Mls	% Specific Release (a) 20:1
BALB/c Con A	d	b	8
BALB/c LPS	d	b	-2
DBA/2 Con A	d	a	-5
DBA/2 LPS	d	a	-1

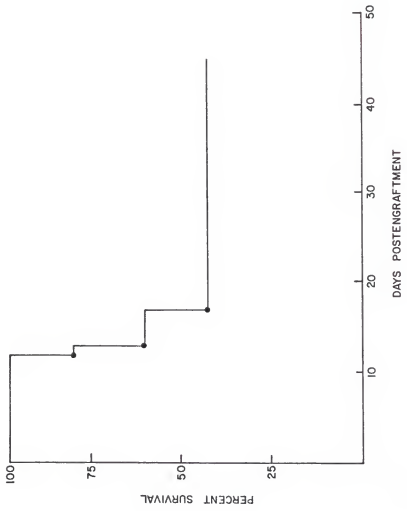
a) This test was performed in a 6 hr assay. GVH reactivity was induced in sublethally irradiated DBA/2 mice with BALB/c splenocytes. On day 6, the spleens were collected, made into a single cell suspension and cultured overnight in interleukin 2 prior to use. The GVH primed cells were assayed at a 20:1 effector to target ratio.

Table 33.
CML reactivity of BALB/c anti-DBA/2 cells generated in GVH

Target cells	H-2	Mls	% Specific Release (a) 24 hrs	48 hrs
BALB/c	d	b	0	0
DBA/2	d	a	0	-1
B10.D2	d	b	0	-1
B10.BUA16	w	b	-1	-3

a) This test was performed in either a 24 or 48 hr assay. GVH reactivity was induced in sublethally irradiated DBA/2 mice with BALB/c splenocytes. On day 6, the spleens were collected, made into a single cell suspension and used the same day as they were removed from the animal. The GVH primed cells contained 15% lymphocytes and 85% PMNs. These cells were then used at a 50:1 effector to target ratio.

Figure 27. Effect of minor histocompatibility antigens on survival in AKR/J mice reconstituted with CBA/CaH cells. Forty million CBA/CaH splenocytes were injected via the tail vein into sublethally irradiated AKR/J mice. Mortality was scored on the day the mouse died.


100
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PERCENT SURVIVAL
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20
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40
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DAYS POSTENGRAFTMENT

Histopathological examination of the AKR mice suffering from acute GVHD revealed an identical disease picture as described earlier (Section 3.2 F). In addition, when the spleen cells of these GVHD affected mice were tested on day 10 for cytolytic activity, CML was exhibited on AKR targets, with weaker activity on DBA/1 and DBA/2 target cells (Table 34). Although the CML activity appears to be directed against Mls^a, this may not be the target since CBA/J, which is now thought to possess the Mls^a determinants, was not killed. In addition, no CTL activity against Mls^a was observed in the BALB/c anti-DBA/2 system. Thus it is likely that the CBA/CaH cells are detecting another antigen which is present on AKR cells.

K. Ability of primed lymphocytes to cause mortality in sublethally irradiated mice

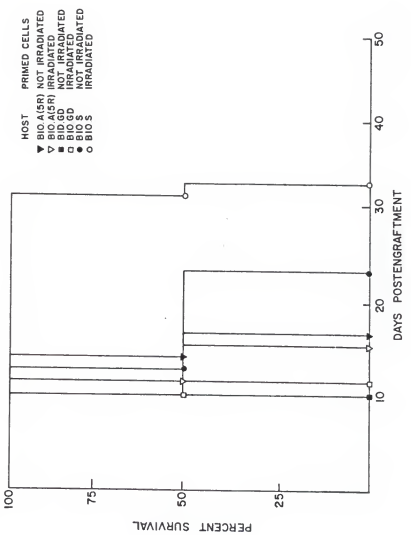
In an attempt to determine whether GVH primed lymphocytes could be passaged into secondary hosts, it was discovered that lymphocytes primed either in in vitro or in vivo have the ability to cause mortality in both syngeneic and allogeneic sublethally irradiated animals. This is presented in Figure 28 for B10.A(5R), B10.GD and B10.S mice which were sublethally irradiated and injected i.v. with 1×10^6 (B10.A(4R) x B10.GD)F_1 anti-B10 primed cells generated in vivo. As can be seen, these

Table 34.
CML reactivity of primed CBA/Ca anti-AKR cells generated in GVH

Target cells	H-2	Mls	Specific Release (a) 40:1	20:1
AKR	k	a	33	39
DBA/1	q	a	16	12
DBA/2	d	a	13	10
CBA/J	k	d	7	8
B10.BR	k	b	5	-
CBA/Ca	k	b	0	11

a) This test was performed in a 6 hr assay. GVH reactivity was induced in sublethally irradiated AKR mice with CBA/Ca splenocytes. On day 10, the spleens were collected, made into single cell suspensions and cultured overnight in interleukin 2 prior to use. The GVH primed cells were assayed at either 40:1 or 20:1 effector to target ratios.

Figure 28. Ability of 10^6 (B10.A(4R) x B10.GD)F_1 anti-B10 primed cells to cause mortality in sublethally irradiated mice. Host animals: B10.A(5R), B10.GD or B10.S were sublethally irradiated with 650 rads and then reconstituted with 10^6 (B10.A(4R) x B10.GD)F_1 anti-B10 primed cells either X-irradiated or not. Mortality was scored on the day the mouse died.


HOST PRIMED CELLS
▼ BIO A(SR) NOT IRRADIATED
▽ BIO A(SR) IRRADIATED
■ BIO A(R) NOT IRRADIATED
□ BIO GD IRRADIATED
● BIO S NOT IRRADIATED
○ BIO S IRRADIATED
PERCENT SURVIVAL
100
75
50
25
10
20
30
40
50
DAYS POSTENGRAFTMENT

anti-K^b, I-A^b cells proved lethal for both the allogeneic mice B10.A(5R) and the syngeneic mice B10.GD, with death occurring between days 10 and 17. The primed cells could even be irradiated with 2000 R of irradiation and yet still cause mortality in these animals. Third party mice, such as B10.S, also died between days 15 and 22; however irradiation of the primed cells increased survival time of the B10.S mice even though they eventually died by day 32. It should be noted that the primed cells had no effect when injected into nonirradiated mice, either syngeneic or allogeneic.

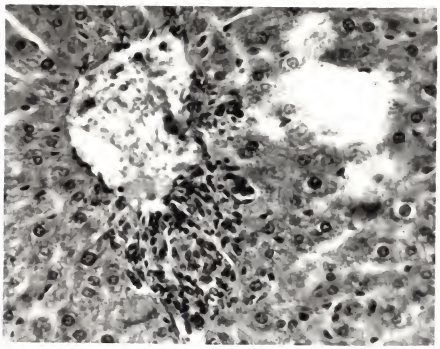
In a follow up study using B10.RIII anti-B10.M primed cells generated in vitro, similar results also occurred. Both syngeneic and allogeneic mice died between days 10 to 20 after engraftment. Supernates of the primed cells also caused mortality in sublethally irradiated mice. Interestingly, if primed cells were cultured overnight in fresh medium, this supernate did not have the ability to cause mortality in sublethally irradiated mice. In addition, normal unirradiated mice were not susceptible to mortality induced by either the primed cells or culture supernate even up to 3 months after injection. Neither the supernate nor the cells could induce pathological effects to an embryonic mouse fibroblast culture, indicating that such factors as cytopathic viruses, were not playing a role in mortality.

L. Histology and pathology of secondary disease

Mice receiving primed cells from the previous experiment were examined histopathologically on day 10. When B10.RIII anti-B10.M primed cells were injected into sublethally irradiated B10.M mice, the normal histology of acute GVHD was observed in the liver. Perivascular cuffing and leukocytic infiltrates associated with the veins and bile ducts were present together with the parenchyma damage (Figure 29). In the lungs of these animals some edema and mild hypercellularity was also observed (Figures 30 and 31). In marked contrast, when B10.RIII anti-B10.M cells were injected in irradiated syngeneic hosts, a different pathology was observed. The lungs were markedly more hypercellular with much more edema and congestion (Figure 32). In addition, the spleen also showed marked hypercellularity (Figure 33). Presumably, large numbers of the injected cells became trapped in these two organs. In the liver, leukocytic infiltrates were found infrequently. Such infiltrates, when found, appeared in the parenchyma *per se* and not along the central vein or bile ducts (Figures 34 and 35). This appears to be similar to a hepatitis type lesion, and not like the pathology observed in the livers of hosts with allogeneic GVH disease.

Figure 29. The liver of a B10.M animal undergoing GVHD from primed lymphocytes: B10.RIII anti-B10.M. This liver shows perivascular cuffing of invading leukocytes. The leukocytes are radiating from a central vein and appear to be invading the parencyma.

Figure 30. The lung of the B10.M animal previously examined. This lung shows little abnormality in it. A little edema is seen but this is similar to that seen in the irradiated control.



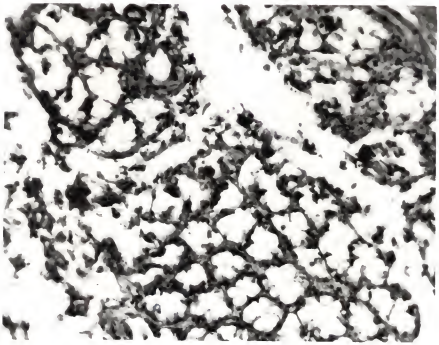
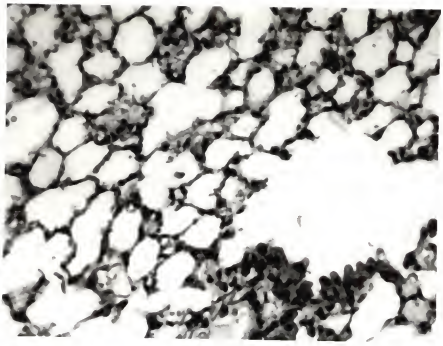


Figure 34. The liver of a sublethally irradiated B10.RIII mouse reconstituted with primed B10.RIII anti-B10.M lymphocytes. Leukocytic infiltrates are commonly found in these treated animals. No perivascular cuffing is observed. The parenchyma of the liver appears slightly edematous. However, unlike the lesions of GVHD there does not appear to be any massive amounts of necrosis.

Figure 35. A higher magnification of the previous liver. The parenchyma appears edematous, but is essentially intact. The hepatocytes show well defined membranes. A leukocytic infiltrate is observed at the upper left corner, but this is a different type of infiltrate as compared to figure 10. Necrosis is not readily apparent.



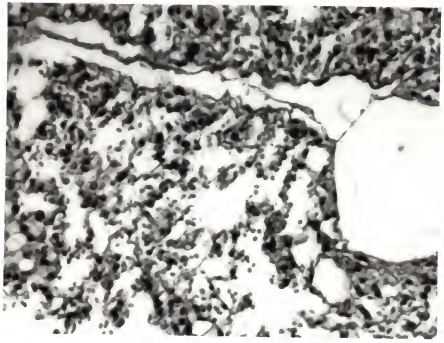


Figure 33. The spleen of the animal previously examined. The spleen is hyerchromatic due to the hypercellularity, massive amounts of mononuclear cells are present. Little normal architecture is seen due to the crowded conditions. The pathological change is much different from that seen in Figure 15.

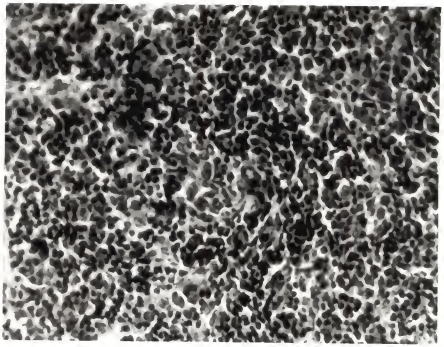
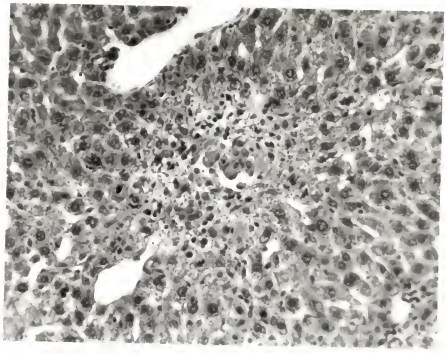
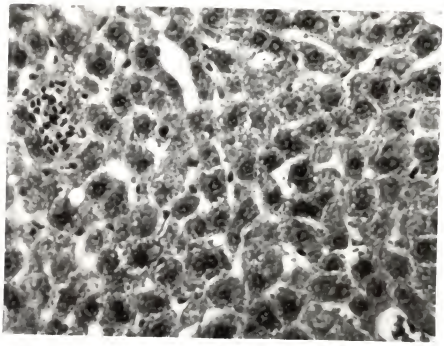


Figure 31. The lung of a normal irradiated mouse 14 days after irradiated. The alveolar walls appear thin with little edema. The alveolar sacs are evenly spaced. Very few mononuclear cells are found in the alveoli. No gross morphological changes are seen.

Figure 32. The lung of a B10.RIII mouse reconstituted with primed B10.RIII anti-B10.M cells day 14. The alveolar walls are substantially thickened and congested. The tissue is edematous, but there is no cellular exudate present. Monocytic cellular infiltrates can be discerned in the alveolar walls. This process is indicative of interstitial pneumnitis.



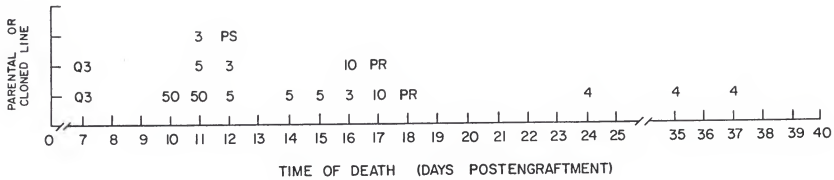


M. Mortality induced by anti-I-A^q long term cultured T cell lines and cloned T cell lines

The long term anti-I-A^q cell lines (B10.RIII x B10.AQR)F_1 anti-B10.T(6R) and (B10.S x B10.AQR)F_1 anti-B10.T(6R) have been maintained for over two years by Dr. A.B. Peck through sequential restimulations (presently 42 restimulations) with B10.T(6R) cells. At the time of the 25^{th} restimulation, the (B10.RIII x B10.AQR)F_1 anti-B10.T(6R) line was cloned using limiting dilution procedures. Of fifty growth positive microtiter plates with "presumed" clones which were transferred to culture flasks for expansion, ten grew well enough for study. Analyses of the reactive patterns of these cells are presented elsewhere (105). The parental lines and the cloned lines have been maintained without the use of exogenous IL 2. The cells are Lyt 1+2-, react strongly in secondary MLRs, are noncytotoxic and produce T helper lymphokines.

Reconstitution of (B10.T(6R) x B10.AQR)F_1 with 10^6 parental or cloned T cells has been found to induce severe GVH like disease resulting in death of the host between days 10 to 18 (Figure 36). One cloned line, 4, however, proved highly inefficient in inducing lethal GVH disease. This cell line is known to react primarily against the I-A^p molecule cross reacting against the I-A^q gene product (105). Each host animal displayed wasting disease, diarrhea and

Figure 36. Mortality in sublethally irradiated (B10.T(6R) x B10.AQR)F_1 hosts induced by anti I-A^d long term cultured T cell lines and clones. (B10.T(6R) x B10.AQR)F_1 mice were sublethally irradiated with 650 rads and then reconstituted with 10^6 long term cultured T cell lines [PS, parental (B10.S x B10.AQR)F_1 anti-B10.T(6R) line; PR, parental (B10.RIII x B10.AQR)F_1 anti-B10.T(6R) line or cloned lines (3,4,5,10 and 50)] via the tail vein. B10.AQR mice were sublethally irradiated and reconstituted with hemiallogeneic cells of clone 3 (Q3). Mortality was scored on the day the mouse died.


PARENTAL OR CLONED LINE
3 PS
Q3
5 3
10 PR
Q3
50 50 5
5 5 3 10 PR
4
4 4
0 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 35 36 37 38 39 40
TIME OF DEATH (DAYS POSTENGRAFTMENT)

hypothermia. Histologically, the liver, lungs and intestines revealed the pathology of GVH disease. Somewhat unexpectedly, however, these same cloned lines have been found to be capable of inducing disease in sublethally irradiated B10.AQR hosts. However, histopathological examination has revealed a greater involvement of the lungs, with concommitant less involvement of the liver and intestine. The common lesions of GVHD are not present in these animals. These latter points suggest the disease process may be different and has nonspecific components.

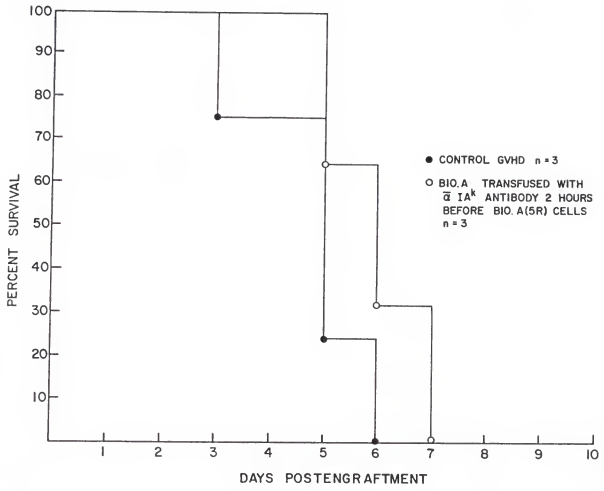
Not every long term primed cell line is capable of causing mortality in sublethally irradiated mice. For example, the B10.D2 anti-B10 line produced by Dr. B. Elliott and maintained by Dr. A. Kimura, when injected into B6 mice no mortality was observed a 60 day observation period (data not shown). This cell line does not produce large amounts of detectable soluble lymphokines (Peck and Kimura, personal communication); thus, the lack of mortality may be due to the fact that lymphokine production is limited and as a result no lymphokine mediated responses are elicited. However, this hypothesis remains at best speculative.

3.3 Attempts to Modify GVHD

A. Attempts to prevent lethal GVHD using concentrated monoclonal antibodies directed towards the host's I-A molecule

Results obtained from the GVH and PLT data suggested that the I-A molecule is crucial in the development of lethal GVHD. It was decided, therefore, to determine if blocking the recognition of the I-A molecule of the host by using concentrated monoclonal antibody would modify subsequent GVH. The system examined was as follows. One milliliter of the purified monoclonal antibody (10.2.16 generoulsy provided by Dr. E. Wakeland) at a concentration of 1 mg/ml was injected i.v. into sublethally irradiated B10.A mice 2 hours prior to receiving the B10.A(5R) donor cells. Monoclonal antibody 10.2.16 binds the $I\text{-}A^k$ molecule expressed in B10.A mice. Survival was then used to indicate whether the antibody had any beneficial effects. The results, shown in Figure 37, indicate that both the control GVH animals and the experimental animals succumbed at the same rate. Histological studies revealed that both sets of mice had similar lesions. Under the present test conditions, it would appear that GVHD was unable to be controlled by this protocol. This combination differed genetically at both $I\text{-}A^k$ and K^k. This K difference, while insuring a lethal GVHD, may have distorted the final interpretation.

Figure 37. Inability of concentrated (ascites) anti-I-A^k antibody (10.2.16) to prevent lethal GVHD in sublethally irradiated B10.A mice reconstituted with B10.A(5R) splenocytes. This figure demonstrates the survival curves of two sets of 3 mice which were sublethally irradiated and reconstituted with B10.A(5R) splenocytes: a K^k I-A^k disparate GVH. Two hours before receiving the allogeneic cells the experimental group of mice receieved 1 ml of concentrated anti-I-A^k monoclonal antibody (10.2.16). As can be seen no differences exist between the 2 survival curves.


100
90
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30
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10
PERCENT SURVIVAL
1
2
3
4
5
6
7
8
9
10
DAYS POSTENGRAFTMENT
● CONTROL GVHD n = 3
○ BIO.A TRANSFUSED WITH
$\overline{a}$ IA^k ANTIBODY 2 HOURS
BEFORE BIO. A(5R) CELLS
n = 3

B. Attempts to prevent lethal GVHD using neonatal splenocytes

i. Inhibition of acute lethal GVH disease using CBA/J newborn spleen cells

The experimental protocol utilized in this study to test for the ability of newborn spleen cells to suppress lethal GVH disease in irradiated host mice reconstituted with allogeneic adult cells was as follows. Donor adult spleen cells were incubated 24 hours in the presence or absence of newborn spleen cell populations prior to engraftment in sublethally irradiated host mice. Newborn spleen cell populations were suspensions of whole spleen cells which had incubated in culture plates 12 hrs. After 18 hrs of coculturing, 30×10^6 viable cells were injected i.v. into host animals. Control reactions included donor adult cells cultured in medium alone or cocultured with adult spleen cells syngeneic with the newborn cells.

Results from two sets of experiments in which CBA/J newborn cells were cultured with adult B10.BR cells prior to engraftment of semi-allogeneic (B10 x B10.BR)F_1 or allogeneic B6 hosts are presented in Tables 35 and 36, respectfully. Host animals receiving adult B10.BR cells or B10.BR cells cocultured with adult CBA/J spleen cells died of lethal GVH disease between days 6 and 12. In contrast, only 33% of the host mice receiving B10.BR cells cocultured

with CBA/J newborn spleen cells at a ratio of 10:1 died within 12 days, while 66% of the mice showed long term survival. Similar survival rates were observed for mice receiving B10.BR cells cocultured with CBA/J newborn spleen cells at ratios of 14:1 and 28:1.

Data presented in Tables 35 and 36 reveal a number of additional points. First, newborn spleen cells alone were unable to induce lethal GVHD. Second, the suppression of GVH by the newborn spleen cells could be diluted out at adult to newborn cell ratios greater than 56:1. Third, CBA/J newborn thymus cells were unable to suppress the GVH reactivity of adult cells. Fourth, whereas AKR newborn spleen cells were as effective as CBA/J cells in suppressing subsequent GVH in B6 hosts, (B10.BR x SWR)F_1 newborn spleen cells proved inactive in (B10 x B10.BR)F_1 hosts.

ii. Histopathology of the experimental and control host animals

All mice which died were examined histologically to verify the presence of GVH disease. In addition, mice showing long term survival were occassionally sacrificed at various times and examined histologically for signs of GVH disease. In control and experimental animals which died or were presumed to die prior to day 14 to 15 postengraftment, classical symptoms of GVHD were noticeable in histological

Table 35.
Suppression of lethal GVHD by newborn spleen cells in (B10 X B10.BR)F_1 host animals reconstituted with semi-allogeneic adult B10.BR spleen cells.

Responding cell population	Survival Times (a)
B10.BR	7, 8, 9, 10, 10, 10, 10, 10 [9]
B10.BR + CBA/J Adult cells	7, 9, 12 [9]
B10.BR + CBA/J Newborn spleen cells	8, 12, 50+, 50+, 50+, 50+ [45+]
Newborn CBA/J spleen cells	50+, 50+, 50+ [50+]
B10.BR + Newborn (B10.BR x SWR)F_1 spleen cells	7, 7, 7, 8, 8, 9, 9, 9, 9, 10, 10, 10, 10, 11 [9]

a) The number represents the day postengraftment that a given animal died. Underlined dates represent mice that have lived considerably longer than control GVH animals. The number within [ ] indicates the mean survival time of a given series of animals. The symbol + indicates that those animals were still living after that day and did not appear to suffer from GVHD.

Table 36.
Suppression of lethal GVHD by newborn spleen cells in B6 host animals reconstituted with allogeneic adult B10.BR spleen cells.

Responding cell population	Survival Times (a)
B10.BR	6, 7, 8, 8, 10, 12 [9]
B10.BR + CBA/J Adult cells	7, 8, 11 [9]
B10.BR + CBA/J Newborn thymus cells	5, 6, 6, 7 [6]
B10.BR + CBA/J Newborn spleen cells	
10:1 (b)	10, 14, 25+, 25+, 72, 74 [36+]
14:1	9, 30+, 30+, 40+ [27+]
28:1	8, 30+, 30+, 40+ [27+]
56:1	10, 11, 30+, 30+ [20+]
100:1	7, 9, 10, 10, 12 [10]
B10.BR + CBA/J Newborn spleen supernate	6, 7, 16 [10]
B10.BR + AKR Newborn spleen cells	8, 20+, 20+, 20+ [17+]

a) The number represents the day postengraftment that a given mouse died. Underlined dates represent mice that have lived considerably longer than control GVH animals. The number within [ ] indicates the mean survival time of a given series of animals. The symbol + indicates that those animals were still living after that day and did not appear to suffer from GVHD.
b) The ratio of adult:newborn cells used in a given experiment.

sections beginning on day 5 to 6. Such animals exhibited wasting, diarrhea, a hunched posture and hypothermia.

Of the long term surviving animals, approximately 30% developed symptoms of chronic GVHD. Between days 35 and 50 postengraftment, these mice became lethargic, developed skin lesions, and showed some fur loss with associated redness of the skin (Figure 38). Histologic examination revealed leukocytic infiltration in the dermis of the affected areas. In addition, the liver often showed abnormal histology, including a "punched out" appearance similar to chronic hepatitis, loss of hepatocytes, and disruption of the liver chord pattern (Figures 39 and 40). No gross abnormalities were seen in either the spleen, kidney or intestines.

iii. Functional reactivity of donor cell populations after initial period of culturing

To control for the possibility that the culturing of adult cells with CBA/J newborn spleen cells led to a functional inactivation, these mixed donor cell populations were tested for their mitogenic responsiveness to the T cell mitogen Con A and the B cell mitogen LPS. As shown in Table 37, adult B10.BR cells cultured alone, as well as B10.BR cells cultured with newborn CBA/J cells responded strongly

Figure 38. The skin of a CBA/J newborn suppressed mouse day 72. This skin belonged to the mouse examined in the previous micrograph. By day 25 this mouse began to lose some of its fur and where it did lose it, the skin was red. In this figure leukocytic infiltrates are found in the dermis (lower left corner), while the epidermis appears normal and acellular. From this finding it appears chronic GVHD exists in the animal.

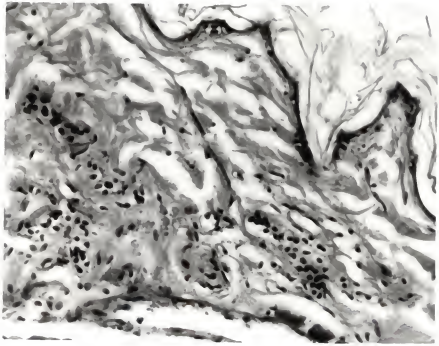
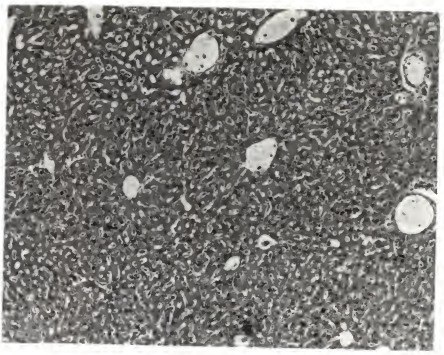


Figure 39. The liver of a CBA/J newborn GVH suppressed mouse day 72. This (B10 x B10.BR)F_1 animal was reconstituted with B10.BR splenocytes treated with CBA/J newborn suppressor cells. This animal survived much longer than did the GVH control mice who died by day 14. This liver demonstrates a "punched out" lesion. Here numerous vacoules are seen within the vacoule is a nucleus.

Figure 40. A higher magnification of the previous micrograph. This micrograph reveals the heterogeneity of the nuclear remants. The liver is obviously different from the acute GVHD liver pathology seen in Figure 10.



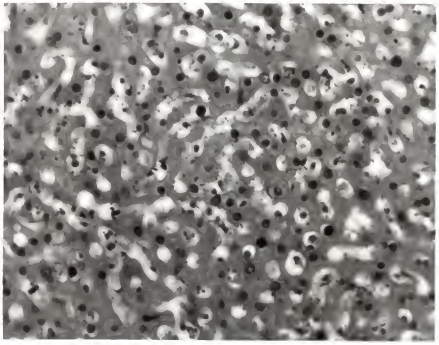


Table 37.
The ability of B10.BR splenocytes incubated with CBA/J newborn splenocytes to respond to mitogens.

cell population	^{3}H-TdR Incorporation CPM±SD (a)		
	no addition	Con A (b)	LPS (c)
B10.BR untreated (d)	5977± 899	45398±13849	64360±6330
B10.BR cultured alone for 18 hr (e)	9569± 908	96703±8313	93245±3892
B10.BR with CBA/J NB cocultured 18 hrs (f)	13067±1642	94637±1540	90382±3493
Newborn splenocytes cultured alone 18hrs (g)	2059±461	2314± 609	2302± 286

a) Cultures were harvested at 48 hrs following a 12 hr pulse with 1 μCi of ^{3}H-TdR.
b) Concanavalin A dose was 5 μg/ml.
c) Lipopolysaccharide dose was 100 μg/ml.
d) B10.BR splenocytes were removed from the animal immediately before use.
e) 10^8 B10.BR splenocytes were incubated for 18 hrs in untreated media.
f) 10^8 B10.BR splenocytes were incubated with 30 x 10^6 CBA/J newborn splenocytes for 18 hrs in media.
g) 30 x 10^6 newborn splenocytes were incubated for 18 hrs in media.

toward both Con A and LPS. Thus, the donor cell populations were still functional at the time of injection into the host mice.

iv. Functional reactivity of lymphocytes derived from long term surviving host mice

As demonstrated earlier, protocols have been described which permit functional testing of cells derived from organs undergoing severe lethal GVH reactivity. Using these procedures, it was possible to examine the proliferative and cytolytic reactivities of cells residing in the organs of control and experimental mice. Spleen cells prepared from control mice exhibiting signs of severe GVHD were removed on day 5 to 6 and cultured overnight before using as responding cells in PLT assays. Similarly, spleen cells were prepared on days 7 to 8 and 25 to 26 from experimental animals showing signs indicative of long term survival. As presented in Table 38, cells from the GVH control mice responded against B6, B10.A(3R) and B10.A(5R) cells expressing the $I\text{-}A^b$ derived gene product similar to the F_1 hosts. In contrast, cells obtained from the long term surviving mice failed to respond to any of the stimulating cells tested. Similar results were found with cells removed from B6 host mice (Table 39). In this case, however all the cell populations were tested and found to be activated by the

Table 38.
The inability of lymphocytes obtained from newborn CBA/J suppressed GVHD to respond in a PLT.

Stimulator Strain	H-2 Genetics K	A	J	E	D	^{3}H-TdR Incorporation CPM±SD Control GVH primed cells (a,b)	CBA/J newborn suppressed GVH cells (a,c) Day 7	Day25
none	-	-	-	-	-	829± 115		3349±175
B10.BR	k	k	k	k	k	753± 69	1206± 36	2785±457
B6	b	b	b	b	b	24828±1195	3043±808	4239± 85
B10.MBR	b	k	k	k	q	1111± 171		1454± 86
B10.A(5R)	b	b	k	k	d	16920± 267		5563±324
B10.A(3R)	b	b	b	k	d	21088±1515		3373±378
B10.GD	d	d	b	b	b	5163± 592		3295±500
B10.A(4R)	k	k	b	b	b	1081± 209		2979± 92
B10.A(2R)	k	k	k	k	b	981± 9		2179±495

a) PLTs were harvested at 48 hrs following a 12 hr pulse with 1 μCi of ^{3}H-TdR.

b) GVH reactivity was induced in sublethally irradiated (B10 x B10.BR)F_1 mice with B10.BR splenocytes which were cultured overnight prior to use. On day 5, the spleens were collected, made into a single cell suspension and cultured overnight prior to use.

c) Suppressed GVH reactivity was induced in sublethally irradiated (B10 x B10.BR)F_1 mice with B10.BR splenocytes which were cocultured with CBA/J newborn splenocytes overnight prior to use. On day 5, the spleens were collected, made into a single cell suspension and cultured overnight prior to use.

Table 39.
Responsiveness of splenocytes from CBA/J newborn splenocyte mediated acute GVHD suppressed mice to react to various stimulants.

Stimulant	^{3}H-TdR Incorporation (CPM±SD) of Responding Cells derived from: Irradiated B6 mouse (a)	B6 reconstituted with CBA/J NB (b)	B6 reconstituted with B10.BR + CBA/J NB (c)	B6 reconstituted with B10.A(5R) + CBA/J NB (d)
none	3063±1225	2934±1835	3166±1084	9436±3492
Con A (e)	91987±5140	94241±8183	26761±8114	50441±1534
LPS (f)	37468±9245	46016±4099	24491± 225	66061±1606
B6 (g)	1077±1178	2360± 571	1077± 45	1512± 345
B10.A(5R)	10860± 199	3481± 512	2882± 831	1198± 93
B10.BR	16550± 229	1775± 49	1405±1257	2091± 33
CBA/J	25790± 128	689± -	283± 83	1623± 225
B10.S	11131± 524	1959± 342	2671± 356	5135± 667

a) A B6 mouse was sublethally irradiated with 650 r. The spleen was removed and used in the assay 30 days after the irradiation.
b) A B6 mouse was prepared as in a), except 10^6 CBA/J newborn splenocytes were injected after the irradiation. The spleen was extracted and used in the assay 30 days after engraftment.
c) A B6 mouse was prepared as in a), except 30 x 10^6 B10.BR splenocytes which were cocultured with CBA/J newborn splenocytes before injection were infused into the mouse. The spleen was extracted and used in the assay 30 days after engraftment.
d) A B6 mouse was prepared as in a), except 30 x 10^6 B10.A(5R) splenocytes which were cocultured with CBA/J newborn splenocytes before injection were infused into the mouse. The spleen was extracted and used in the assay 30 days after engraftment.
e) The dose of Concanavalin A was 5 µg/ml. This culture was pulsed with 1 µCi of ^{3}H-TdR for 12 hrs on day 2 of the reaction.
f) The dose of lipopolysaccharide was 100 µg/ml. This culture was pulsed the same time as the Con A culture.
g) Primary MLRs were established with the following splenocytes: B6, B10.A(5R), B10.BR, CBA/J and B10.S. These cultures were pulsed with 1 µCi of ^{3}H-TdR for 12 hrs on day 4 of the reaction.

mitogens Con A and LPS. By day 60, those mice which did not develop chronic GVHD responded to third party alloantigens as well as towards the donor cells, indicating that the donor cells had been rejected and that the host animal has recovered from the radiation.

The cell populations isolated from the CBA/J newborn mediated GVHD suppressed B6 hosts engrafted with B10.BR cells were also tested for their cytolytic activity in the CML assay (Table 40). Spleen cells from both the control and experimental hosts were placed in culture and stimulated with interleukin 2 for 7 days. Cells from the experimental mice showed large loss in numbers due to death. Furthermore, when examined in CML, these cells failed to kill either the B6 or B10.BR target cells. In contrast, cells from the control animals undergoing severe GVHD not only expanded in the presence of the interleukin 2, but also proved capable of lysing B6 target cells, syngeneic with the host mice.

Experimental animals which have survived considerably longer than the GVH control animals were tested for chimerism by determining the presence of H-2 antigens of the donor ($H\text{-}2^{k}$ or $H\text{-}2^{i3}$) and/or the host ($H\text{-}2^{b}$). The splenocytes from a sublethally irradiated B6 mouse were lysed only by the anti-$H\text{-}2^{b}$ alloantisera plus complement

Table 40.
The inability of cells obtained from a newborn CBA/J suppressed GVHD to respond in a CML. (a).

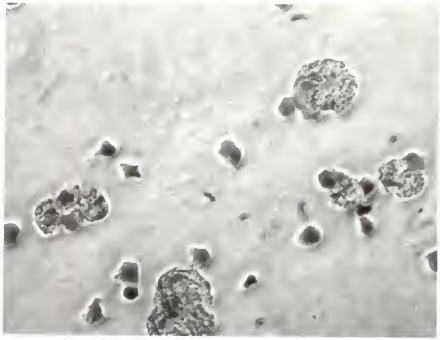
	% Specific Release (b)				
	GVH Control		CBA/J Newborn suppressed		
Target cells	18:1	6:1	17:1	8:1	4:1
B6 Con A blasts	31	15	0	8	1
B10.BR Con A blasts	2	-12	-3	-4	-4

a) The splenocytes from (B10 x B10.BR)F_1 mice reconstituted with B10.BR splenocytes supplemented with CBA/J newborn splenocytes were removed from the animals on day 15 and were fed purified interleukin 2 every second day for 1 week.
b) This test was performed in a 6 hr assay. The effector to target ratios were: 17:1, 8:1 and 4:1.

(96%). The cells from a B6 mouse reconstituted by the B10.BR cells treated with the CBA/J newborn suppressor factor were killed by both the anti-H-2^b antisera as well as the anti-H-2^k antibody, 66% and 33% respectively. This evidence would suggest that the reconstituted animal was a chimera. Chimerism was also demonstrable in a B6 mouse reconstituted with B10.A(3R) splenocytes treated with cells from CBA/J newborn splenocytes. Here 73% of the lysable cells were killed by the anti-H-2^b antisera plus complement, while 24% of the cells were killed by the anti-H-2^k antibody plus complement. Since this anti-H-2^k antibody was a monoclonal antibody with the K^k specificity, only CBA/J derived cells (H-2^k) were lysed and the donor cells from B10.A(3R) which are K^b positive were not lysed.

One cell which constantly appeared to expand in interleukin 2 maintained cultures was a basophil/granulocyte like cell (Figure 41). Interestingly, this cell appeared to be clustered with lymphoid cells. From these functional studies (Tables 36 and 37) it appears that these mice have no anti-self reactivity and that the suppression due to the CBA/J suppressor cells is complete as judged by these two functional tests used.

Figure 41. A single cell suspension prepared from a (B10 x B10.BR)F_1 spleen 25 days after reconstitution with B10.BR cells and newborn CBA/J splenocytes. These cells were cultured for 10 days in the presence of interleukin 2. Numerous large basophilic cells containing granules are seen. These cells were present when the single cell suspension was initially prepared from the GVH suppressed animal. These cells were taken from an animal undergoing chronic GVHD.



v. Genetic Restrictions in the ability of CBA/J newborn cells to suppress lethal GVHD

In light of the apparent nonspecific suppression of adult immune reactivity in vitro by newborn spleen cells, it was surprising to find that not all newborn spleen cells were capable of suppressing GVHD in the donor/recipient combinations, e.g., the B10.BR, SWR or the (B10.BR x SWR)F_1 combination (Figure 35). Examination of this question in several genetic situations now suggests that there are genetic restrictions governing the ability of newborn spleen cells to elicit a suppression of subsequent GVHD. This point is clearly illustrated with a few representative experiments presented in Table 41. The first restriction that becomes apparent is the requirement for histocompatibility (most likely at the 17^{th} Chromosome) between the newborn suppressor cell population and the donor cell population. Thus, whereas CBA/J newborn cells suppress adult cells expressing the H-2^k haplotype, they are incapable of inhibiting adult cells of unrelated adult cells of unrelated H-2 haplotypes, e.g., B10.WB (H-2^{ja}) hosts engrafted with B6 (H-2^b) cells, as shown in Table 41 set 1.

Table 41.
The genetic restrictions in the suppression of lethal GVHD by newborn spleen cells in host animals reconstituted with allogeneic adult spleen cells.

Reconstituting cells	Hosts	Day of Mortality: (a)
SET 1		
B6	B10.WB	5, 6, 7 [6]
B6 + CBA/J Newborn cells		6, 7, 8 [7]
SET 2		
B10.A(5R)	B6	5, 12, 20 [12]
B10.A(5R) + CBA/J Newborn cells		5, 14, 38+, 38+ [24+]
B10.A(3R)	B6	12, 12, 12, 14, 30. [16]
B10.A(3R) + CBA/J Newborn cells		14, 19, 20, 50+, 50+, 50+ [34+]
B10.A(4R)	B6	7, 11, 12 [10]
B10.A(4R) + CBA/J Newborn cells		11, 11, 11, 11 [11]
B10.A(2R)	B6	4, 5, 10 [6]
B10.A(2R) + CBA/J Newborn cells		5, 5, 5 [5]
B10.MBR	B6	5, 6, 7, 10, 10, 10 [8]
B10.MBR + CBA/J Newborn cells		4, 6, 10, 10, 10, 10, 11, 19 [10]
B10.HTT	B6	7, 7, 8 [7]
B10.HTT + CBA/J newborn cells		7, 7, 8 [7]
SET 3		
B10.D2	B6	10, 12, 13, 16 [13]
B10.D2 + CBA/J Newborn cells		8, 8, 8, 10 [9]
B10.D2 + BALB/c Newborn cells		10, 10, 10, 10 [10]
B10.D2 + SEC Newborn cells		7, 14 [10]
B10.D2 + SEA Newborn cells		26, 45, 46 [39+]
B10.D2 + DBA/2 Newborn cells		10, 12, 25, 60+, 60+, 60+ [38+]
SET 4		
SWR	B6	8, 9, 10, 10, 10 [10]
SWR + SWR Newborn cells		9, 10, 10, 11, 11 [10]

a) Underlined dates represent mice that have lived considerably longer than control GVH animals. The number within [ ] indicates the mean survival time of a given series of animals. The symbol + indicates that those animals were still living after that day and did not appear to suffer from GVHD.

The apparent H-2 restriction observed with CBA/J newborn cells using various strains carrying recombinant H-2 haplotypes derived from the a and b parental haplotypes were also investigated. As presented in Table 41 set 2, CBA/J newborn cells suppressed the GVH reactivity of B10.A(3R) and B10.A(5R) but not of B10.A(4R), B10.A(2R), B10.MBR, B10.D2 and B10.HTT.

A second genetic factor in the suppression of adult GVH reactivity by newborn spleen cells apparently involves a non-H-2 genetic system, which at this time correlates with the expression of the Mls locus. In the examples thus far presented, CBA/J and AKR but not B10.BR, SWR, B6 or (B10.BR x SWR)F_1 newborn spleen cells elicited suppression. Two further examples are presented in Table 41 sets 3 and 4. In the H-2^d haplotype system, DBA/2 and SEA but not SEC or BALB/c newborn spleen cells were able to elicit suppression of adult B10.D2 reactivity in B6 hosts. In the H-2^q haplotype system, DBA/1 and BUB but not SWR newborn spleen cells suppressed GVH activity of adult B10.Q or SWR cells in B6 host mice. Each of the strains which proved capable of eliciting a suppression expresses the strong Mls locus phenotype, ($Mls^{a/d}$), while all the strains which proved incapable of suppressing the GVH reaction express the null allele of Mls, Mls^b.

vi. The presence of newborn spleen cells incapable of suppressing the GVH reactivity of adult cells fails to modify the response of the sensitized donor cells

Cells recovered from mice which have been engrafted with adult plus newborn cells but which still undergo severe GVH disease exhibit the same in vitro response pattern as cells recovered from the spleens of hosts dying from GVH induced by adult allogeneic cells alone. This is shown in Table 42 in which B6 hosts were engrafted with adult SWR cells or adult plus newborn SWR cells. The SWR cells proved incapable of eliciting suppression as they bear the null allele of Mls. The cells from each set of hosts when stimulated in vitro with a panel of secondary MLR stimulating cells exhibited identical response patterns.

C. Attempts to prevent GVHD using newborn spleen supernates

i. Characterization of the newborn supernate

The newborn spleen associated suppressor cell population has been shown by Peeler et al. (106) to elicit its suppressor activity in part through secretion of soluble, culture stable materials, which in turn initiates activation of the suppressor limb of the immune response.

Table 42.
The Ability of lymphocytes obtained from newborn SWR splenocytes supplemented GVHD to respond in a PLT.

Stimulator Cells	^{3}H-TdR incorporation CPM±SD (a) SWR anti B6 (b)	SWR + SWR NB anti B6 (c)
none	419± 259	237± 328
SWR	2718± 210	2136± 45
B6	50668±9675	62687±12826
B10.A(5R)	85819± 81	84995± 1704
B10.A(3R)	57390±1446	61700± -
B10.A(4R)	2250±2678	1767± 1398
B10.GD	14405±3122	10627± 2610
B10.MBR	3646± 296	1846± 17
B10.BR	7169± 257	5928± -

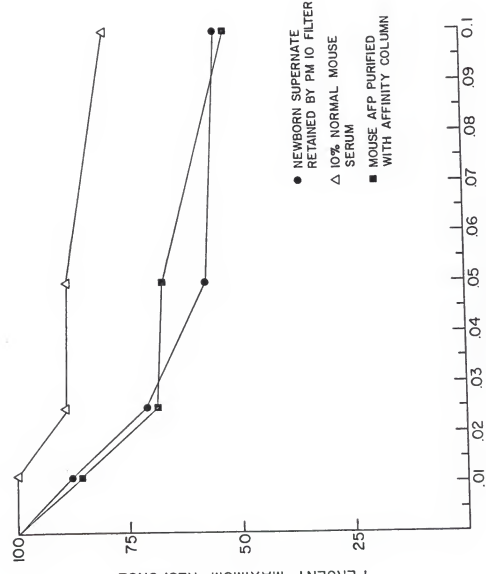
a) PLTs were harvested at 48 hrs following a 12 hr pulse with 1 μCi of ^{3}H-TdR.
b) GVH reactivity was induced in sublethally irradiated B6 mice with SWR splenocytes which were cultured overnight prior to use. On day 5, the spleens were collected, made into a single cell suspension and cultured overnight prior to use in PLT.
c) GVH reactivity was induced in sublethally irradiated B6 mice with SWR splenocytes which were cocultured with SWR newborn splenocytes overnight prior to use. On day 5, the spleens were collected, made into a single cell suspension and cultured overnight prior to use.

Before carrying out in vivo studies with newborn supernate it was decided to further characterize the factor(s) involved. Newborn supernates from various mice were collected and pooled. When 200 mls of the supernate was collected it was concentrated using an Amicon PM 10 filter. This filter allows the compounds with a molecular weight less than 10,000 daltons to pass through, while retaining those substances with a higher molecular weight. When a final volume of 10 mls of the >10,000 M.W. material was obtained, a dose response curve of suppressor activity by this preparation was determined (Figure 42). The dose response curve of this supernate illustrated a bimodal shape with a suppression of 30 to 50% over a range of 1.2% to 10% v/v. No genetic restrictions were seen when these in vitro tests were performed. As a control for this activity, 10% normal mouse serum was used. This was based on the fact that the newborn supernate was generated in 0.5% NMS and these supernates were concentrated 20 times the serum equivalent. When CBA/J newborn supernates were generated in serum free media a similar dose response curve was obtained.

ii. Size profile of the newborn supernate factors

Concentrated newborn supernate was passed through a sephacryl 300 column. The material was collected, each fraction was concentrated 10 fold, filter sterilized, and

Figure 42. Dose response kinetics of various suppressor factors on B10.BR anti-C57BL/6 primary MLR. This illustration shows the dose response curve by adding suppressor factors to a primary MLR. Pooled newborn supernate was collected and passed through an Amicon PM 10 filter, which allows molecules less than 10,000 daltons to escape while retaining larger molecular weight substances. After the supernate was filtered it was dialyzed 3 times in 1000x the volume, in order to allow complete diffusion of all low molecular weight substances. This sample was then assayed in the experiment by diluting the factor as described on the abscissa on a v/v % scale. Since this factor was concentrated in EHAA media containing 0.5% NMS the appropriate control was to use an adjusted concentration of normal mouse serum which represents a 20 fold concentration of 0.5% NMS or 10% NMS. This concentration of NMS also inhibited the primary MLR, but not to the extent as did the newborn supernate. Finally, AFP which was purified by affinity chromatography was also assayed here.


PERCENT MAXIMUM RESPONSE
100
75
50
25
.01
.02
.03
.04
.05
.06
.07
.08
.09
0.1
CONCENTRATION ADDED
● NEWBORN SUPERNATE RETAINED BY PM 10 FILTER
△ 10% NORMAL MOUSE SERUM
■ MOUSE AFP PURIFIED WITH AFFINITY COLUMN

tested for suppressor activity as shown in Figure 43. Three peaks of activity could be found: one low molecular weight substance which eluted off in the last fraction and corresponded to the low molecular weight substance which passed through the Amicon PM 10 filter, a second fraction with a molecular weight similar to bovine albumin, or 60,000 daltons, and a third substance with a molecular weight higher than albumin, but less than the molecular weight of IgG. These compounds were not alpha fetoprotein because by Ouchterlony analysis no line of precipitation was observed, whereas, a line of precipitation was seen when AFP was used (Figure 44). No attempt was made to determine whether this larger suppressor factor was a dimer of the 60,000 dalton protein, due to the scarcity of the protein.

Analysis using a SDS-acrylamide gel has exhibited 2 bands in the range of 6000 to 9000 daltons which are present in cell supernates derived from C3H/HeJ, BALB/c or Bl0.M newborn mice. Neither band was present when the cells from adult mice were cultured for an identical time (Figure 45). In addition, a higher molecular weight species was identified in the 60,000 dalton region of the gel which corresponded to the higher molecular species isolated earlier.

Figure 43. Profile of suppressor activity from newborn supernate passed through a Sephacyrl 300 column to inhibit a primary mixed lymphocyte reaction: B10.BR anti-B10.D2. This figure illustrates the ability of different fractions of the pooled newborn supernate to suppress a primary MLR. There appears to be 3 fractions which have have suppressor activity in them. The first fraction has a molecular weight slightly higher than IgG (150,000), while another fraction has a molecular weight about the same size as albumin (60,000). Finally, the last factor came out in the final void, indicating it was retained very well and has a low molecular weight.

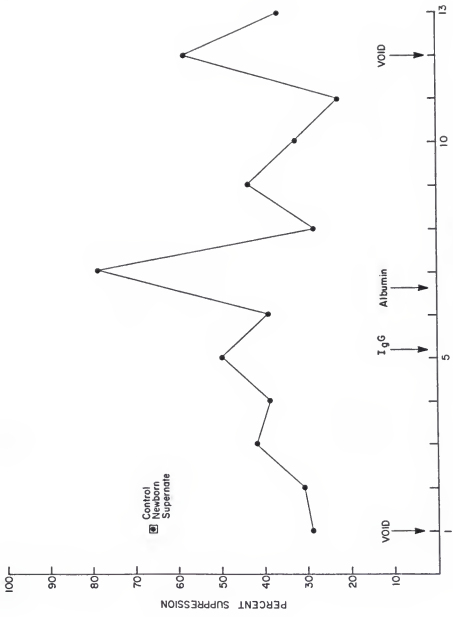

PERCENT SUPPRESSION
100
90
80
70
60
50
40
30
20
10
Control
Newborn
Supernate
VOID
IgG
Albumin
VOID
1
5
10
13
FRACTION NUMBER

Figure 44. Ouchterlony analysis of purified mouse AFP. This precipitation gel shows the ability of rabbit anti-mouse AFP antisera to precipitate mouse AFP. The center well contained the rabbit anti-mouse AFP antisera. Well #1 contained purified standard mouse AFP obtained from Dr. R. Murgita. Well #2 contained mouse AFP collected from the affinity column established by Dr. A. Kimura. Well #3 contained newborn suppressor factor molecular weight 60,000 daltons, well #4 contained low molecular weight newborn suppressor factor which eluted last of the S 300 column. Well number 6 contained the newborn suppressor factor with the molecular weight of 150,000 daltons.

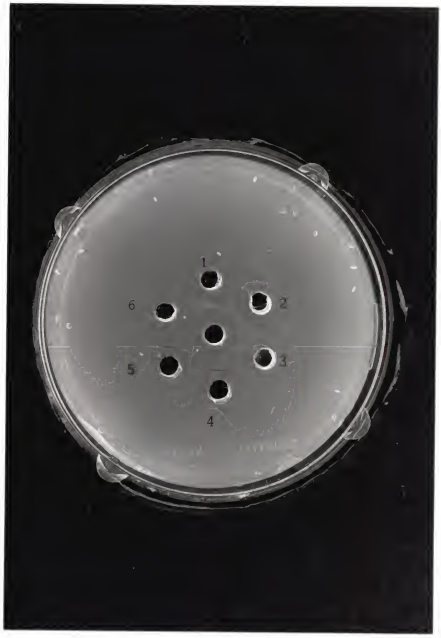
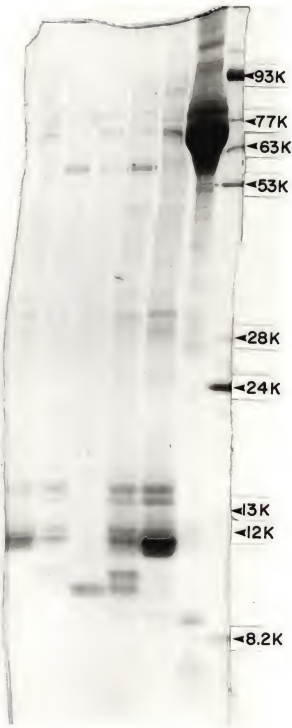

1
2
3
4
5
6

Figure 45. SDS-acrylamide analysis of the newborn factors. Splenocytes from either newborn C3H/He, BALB/c and B10.M mice of adult C3H/He and BALB/c were incubated in PBS for three days. Afterwards the supernates were electrophoresed on a 15 to 30% continous SDS-acrylamide gel. The order of supernates tested were adult C3H/He (lane 1), newborn C3H/He (lane 2), newborn B10.M (lane 3), newborn BALB/c (lane 4) and adult BALB/c (lane 5). Fetal calf serum was a control in lane 6, while different adenovirus proteins served as a control for the molecular weight markers (lane 7).


93K
77K
63K
53K
28K
24K
13K
12K
8.2K

iii. Time of addition studies of the newborn factors

Testing the fractions with the molecular weight of 60,000 daltons and less than 10,000 daltons, it was possible to examine the effect of each in the MLR with respect to time. The results of such a study are shown in Table 43. The higher molecular weight substance is capable of suppressing the reaction up to 48 hrs. In contrast, the low molecular weight substance does not exhibit any time dependency.

The time of pulsing the primary reaction does not alter the results seen in Table 43. The results are identical when the culture is pulsed with ^{3}H-TdR at 96 or 120 hrs. This illustrates that the newborn factors simply do not delay the primary response but do indeed suppress the reaction.

Both of the newborn derived suppressor factors were tested for activity following a) heat treatment at 56 C for 90 min, b) UV light treatment, or c) incubation with 2.5 µg of trypsin for 1 hr at 37 C. The first two treatments did not affect the biological activity of either factor (data not shown); however, treatment with the trypsin altered the activities of both factors (Table 44).

Another aspect of this experiment was an attempt to tentatively identify the nature of these suppressor factors. The MLR was subjected to treatment with either histamine or prostaglandin E_2 to determine what, if any, effect these naturally occurring agents have on this reaction. Histamine

Table 43.
The suppression of primary MLR by adding newborn supernate factors at different times.

Time of addition	^{3}H-TdR Incorporation CPM±SD (a) Control MLR (b)	High MW added (c)	Low MW added (d)
0	25078±1011	14168±1616	15558±2553
18 hours		12841± 394	17097± 999
24 hours		14225± 591	15530± 41
48 hours		17055±1616	18841±1498
72 hours		23413±3383	14491± 98

a) Primary MLRs were harvested at 96 hrs of the reaction following a 12 hr pulse with 1 μCi ^{3}H-TdR.
b) The primary MLR was B10.BR splenocytes reacting against irradiated B10 splenocytes.
c) The high molecular weight substance was added to the primary MLR culture at various time points. The final volume of the factor was 10%.
d) The low molecular weight substance was added to the primary MLR culture at various time points. The final volume of the factor was 25%.

Table 44.
The effect of various substances on a primary mixed lymphocyte reaction.

addition		^{3}H-TdR Incorporation	% of Control Response
none — control (a)		22935±3502	100
Histamine	5.4×10^{-3}M	20855+4078	91
	5.4×10^{-4}M	35186±5284	153
	5.4×10^{-5}M	38466±4183	168
	5.4×10^{-6}M	27040±1569	118
	5.4×10^{-7}M	23420±1246	102
Prostaglandin E_2	10^{-6}M	9352± 660	41
	10^{-7}M	11457± 853	50
	10^{-8}M	18667±3665	81
	10^{-9}M	20148±1128	88
	10^{-10}M	25070±2979	109
CBA/J Newborn High MW Factor		10189± 796	44
CBA/J Newborn High MW Factor Treated with Trypsin (b)		32697±8435	143
CBA/J Newborn Low MW Factor		10743±3003	47
CBA/J Newborn Low MW Factor Treated with Trypsin (c)		18275±3822	80
Trypsin (d)		22717±6235	99

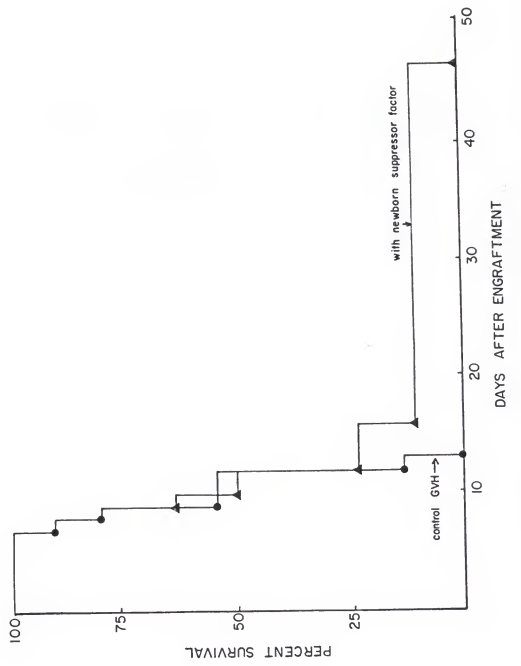
a) A primary MLR was established by B10.BR splenocytes reacting against B6 splenocytes. The cultures were pulsed with 1 μCi of ^{3}H-TdR at 96 hrs for 12 hrs. The various substances were added to the separate wells at the start of the reaction.
b) A sample of the high MW suppressor factor derived from CBA/J newborn splenocytes was incubated with 2.5 μg of trypsin for 2 hrs at 37 C prior to use in this culture.
c) A sample of the low MW suppressor factor derived from CBA/J newborn splenocytes was incubated with 2.5 μg of trypsin for 2 hrs at 37 C prior to use in this culture.
d) An aliqout of the 2.5 μg of trypsin was incubated by itself in a volume equal to that containing the newborn suppressor factors for 2 hrs at 37 C prior to use in this culture.

in a concentration over a range of 5.4×10^{-3} M to 5.4×10^{-7} M demonstrated little if any inhibition of proliferation, but actually enhanced the proliferation at concentrations between 5.4×10^{-4} and 5.4×10^{-6}. Prostaglandin E_2, however, showed a different dose response phenomenon. High doses of this substance (10^{-6} M and 10^{-7} M) suppressed the reaction. It is unlikely that the newborn material is Prostraglandin E_2 because these doses of prostaglandin suppressed MLR reactivity only if added during the first 48 hrs. Additionally, the molecular weight of the proteins seen in the SDS-acrylamide gel was much higher than the molecular weight of prostaglandin E_2.

iv. Effects of the supernate in GVH

To determine if supernate material from newborn spleen cells can also suppress lethal GVHD (B6 x BALB/c)F_1 mice were sublethally irradiated and reconstituted with 40×10^6 BALB/c cells with or without newborn supernate. One half milliliter of the newborn supernate was injected i.v. on days 1,3 and 5. The results of one experiment are shown in Figure 46. Unfortunately, 9 of the 10 experimental hosts died. However, the fact that 1 experimental mouse survived long term may indicate the potential of newborn suppressor factor in preventing acute lethal GVHD.

Figure 46. Lethal GVHD in irradiated (C57BL6 x BALB/c)F_1 mice reconstituted with BALB/c splenocytes. In this experiment groups of 10 sublethally irradiated (C57BL/6 x BALB/c)F_1 mice were treated with BALB/c splenocytes. One group received no further treatment and was considered to be the control GVH reaction. While the experimental group received 0.5 ml of pooled newborn supernate(which was shown to suppress a BALB/c anti C57BL/6 primary MLR) on days 1,3 and 5. All the control GVH mice died by day 12, while in the experimental group 1 mouse survived the first 45 days. This mouse also showed signs of chronic GVHD before it died on day 47.


PERCENT SURVIVAL
100
75
50
25
10
20
30
40
50
DAYS AFTER ENGRAFTMENT
with newborn suppressor factor
control GVH

In addition to using whole newborn supernate, the two isolated fractions were also tested for suppressor activity. Only the low molecular weight factor prolonged survival in the host animals where 1 of 6 experimental animals survived longer than 5 weeks. The high molecular weight material failed to prevent GVHD. In addition, treatment of the donor B10.BR splenocytes with the suppressor factors plus adult CBA/J splenocytes failed to prevent GVHD in the experimental B6 mice. These experiments would suggest that the newborn suppressor cells may be more efficient than the secreted suppressor factors alone in preventing GVHD.

D. Attempts to prevent lethal GVHD using AFP

AFP has been shown to inhibit quite effectively *in vitro* cell mediated reactions (81,82). Since AFP is present in the sera of newborn mice, the question can be raised whether AFP will suppress GVHD. Pregnant mice were sacrificed between days 10 and 13 of fetal gestation. The amniotic fluid was aspirated into a collection flask. After 100 mls of the amniotic fluid was collected it was passed over an affinity chromatography column using rabbit anti-mouse AFP antibody. The antibody and affinity column were prepared by Dr. A. Kimura. The AFP which was eluted off the column was dialyzed and concentrated. This AFP was shown to

bind to the rabbit anti-mouse AFP antibody (Figure 44) and demonstrated a line of identity with an AFP standard obtained from Dr. R. Murgita. This AFP was shown to suppress a primary MLR (Figure 42).

GVHD suppression was attempted in 3 ways. The first method involved incubating virgin B10.BR splenocytes overnight in the presence of a dose of AFP which suppresses the primary MLR (Figure 42). When these cells were injected into sublethally irradiated (B10 x B10.BR)F_1 mice, no GVHD suppression was observed (Table 45). The animals died on days 8,9 and 10, while the control GVHD mice died on days 7,9 and 11.

The second approach was to generate primed cells from a MLR run in the presence of AFP. The concept here was to obtain specific suppressor cells generated by AFP which could subsequently inhibit GVHD. As in the previous approach, the treated mice failed to survive past day 11. Unfortunately, it was impossible to determine whether the mice died from GVHD induced by the donor allogeneic cells or the AFP primed cells which were injected. Thus, this approach was abandoned because of the technical problems.

A third approach was attempted to determine whether systemic levels of AFP in pregnant females was sufficient to prevent acute lethal GVHD. In this protocol pregnant CBA/J mice were sublethally irradiated and reconstituted with 40 x

Table 45.
Lack of GVHD suppression in AFP treated (B10 x B10.BR)F_1 mice.

Reaction:	Day of Mortality: (a)
Control Lethal GVHD (b)	7, 9, 11 [9]
AFP treated mice (c)	8, 9, 10 [9]
AFP treated B10 anti-B10.BR primed cells (d)	10, 10, 11 [10]

a) Day in which the mouse died.
b) B10.BR splenocytes were cultured overnight in media. The following day 40 x 10^6 cells were injected i.v. into sublethally irradiated (B10 x B10.BR)F_1 mice. Using the B10 splenocytes in place of B10.BR cells generated an identical mortality rate.
c) B10.BR splenocytes were cultured overnight in AFP at a dose which was shown to inhibit primary MLRs. The following day 40 x 10^6 cells were injected i.v. into sublethally irradiated (B10 x B10.BR)F_1 mice.
d) B10 anti B10.BR cells were generated in a primary MLR in the presence of AFP by Dr. A.B. Peck. These primed cells were incubated with virgin B10 splenocytes for 2 hrs before being injected into sublethally irradiated (B10 x B10.BR)F_1 mice.

10^6 B10 splenocytes. As shown in Table 46, pregnant animals did not survive longer than non pregnant mice. Thus, this experiment suggests that AFP levels which exists in the third trimester of murine gestation were insufficient to prevent acute GVHD.

Table 46.
Inability of pregnant females to be suppressed from acute GVHD.

Condition:	Day of Mortality:
Non pregnant CBA/J (a)	15, 16 [16]
Pregnant CBA/J (b)	15, 16, 21 [17]

a) Two CBA/J females were sublethally irradiated and reconstituted with 40 x 10^6 B10 splenocytes. Mortality was scored on the day the mouse died.
b) Three CBA/J females which were pregnant (days 14 to 15) were sublethally irradiated and reconstituted with 40 x 10^6 B10 splenocytes. Mortality was scored on the day the mouse died.

DISCUSSION

4.1 Need For Immunosuppression For the Development of GVHD

The first set of experiments (Tables 2 and 3) revealed graft versus host reactions were only observed in immunocompromised individuals. Nonirradiated F_1 mice, which might be expected to be susceptible to parental cell attack, did not develop GVHD, contrary to the classical laws of transplantation (see section 1.1). On the other hand, those animals which were sublethally irradiated and reconstituted with allogeneic cells developed classical GVH conditions such as wasting, diarrhea, lethargy and hypothermia. The dose of irradiation used to suppress the host and make GVHD possible was not sufficient to kill the host via total immunosuppression due to the associated leukocytopenia (Figure 4). The dose used in these studies, then, allows the host's immune system to recover to a degree which protects the host from its environment, yet weakens the host enough so that engraftment of allogenic cells are

generally lethal. This may approximate the situation in bone marrow transplants when bone marrow engraftment does occur.

4.2 The Kinetics of GVHR

In general, GVH reactions followed distinct kinetics (Figure 5). Combinations which differed at the entire H-2 locus produced the mortality within the first 10 days. In the allogeneic reactions, death occured between days 5 to 10 while in semi-allogeneic reactions mortality was observed at days 8 to 14. In class I disparate combinations using K/D or D region differences, mortality occurred between days 10 to 25; however about 25% of the recipients survived longer than 5 months. In I region mismatches, mortality was dependent on the genetic combination. Complete mortality was observed by day 18 in B10.AQR anti-(B10.T(6R) x B10.AQR)F_1 (Figure 5) and (B10 x B10.Q)F_1 anti-B10.MBR (Figure 23). In (B10.MBR x B10.GD)F_1 anti-B10 there was a 30% mortality (Figure 24), while in B10.S(9R) anti-(A.TL x B10.HTT)F_1 no mortality was observed even after 2 months after cell engraftment (Figure 25). Minor histocompatibility mismatches also produced different survival patterns: BALB/c anti-DBA/2 exhibited little if any mortality, B10.D2 anti-DBA/2 demonstrated a more severe reaction with 40% mortality

(Figure 26), CBA/Ca anti-AKR proved to be the most severe of the minor histoincompatible reactions with 80% mortality after 30 days (Figure 27). Thus a wide array of survival patterns were discovered and most interestingly, different functional activities were found in different reactions.

4.3 The Functional Activities of the T Lymphocytes Recovered from GVH Animals

GVH reactions are thought to be initiated by T lymphocytes when these donor cells come into contact with the recipient's histocompatibility antigens. These T cells become activated against the foreign antigens and respond in a number of ways, i.e. by becoming cytotoxic T cells, helper T cells and/or suppressor T cells. I found that T cells generated in GVHR can be recovered with functional activities and these reactivities are highly specific.

It has generally been assumed that the *in vitro* correlate of GVH is the mixed lymphocyte reaction (109). A great deal of information has been obtained using *in vitro* tests. Most of the reactions which occur *in vitro* do correlate with the *in vivo* work, therefore it can probably be inferred that the two situations do have similar factors responsible for reactivities which are observed. For example, it is known that cells which are primed to one antigen will only respond to that antigen in either primed

lymphocyte tests or in cell mediated lympholysis reactions. Those antigens responsible for the initial stimulation of an immune response can then be readily identified. From the *in vitro* tests, it has been possible to test whether the reactions which occur *in vitro* will truly predict what its *in vivo* counterpart should do.

The PLT assays have revealed that class II molecules are responsible for the vast majority of proliferation which is observed *in vitro* by the GVH primed cells. These observations also correlate well with previous studies by Wolters and Benner (110,111). In their work, GVH primed T cells from the spleen and lymph nodes, were capable of being adoptively transferred into normal virgin animals and then were capable of mounting delayed type hypersensitivity reactions against challenges of primary stimulating I region antigens, but not against K/D antigens.

Several possibilities exist to explain this phenomenon. First, suppressor T cells may be present which inhibit K/D proliferation from occurring. This explanation can be eliminated because when GVH primed cells are cocultured with MLR primed cells, which are permissive for K/D proliferation, the resultant proliferation towards K/D antigens occurred (Table 7) illustrating that specific K/D suppressor cells are not present. Another possibility is that incubation of the GVH lymphocytes with contaminating

PMNs may alter the responsiveness of the lymphoblasts. To rule out this hypothesis, GVH primed lymphoblasts can be centrifuged with a higher density Ficoll-Hypaque (d=1.101) which will eliminate all contaminating PMNs and dying cells. When these cells are tested (Table 7) the same proliferative responses are again seen. Thus, it appears that this overall phenomenon is not artifactual, but is indeed real. It would argue that some unknown *in vivo* environmental influences are operating at this level.

The filtration of K/D primed cells in the periphery before these cells arrive in the spleen or lymph nodes may be a possibility, but an unlikely one. The path of injected cells in the host is: blood, lung, lymph node and liver. Early studies by Ford and Gowans (112) have shown that by 12 hrs the majority of donor cells have arrived within the spleen and lymph nodes in the host. The absorption studies performed here indicate that *in vitro* primed proliferative cells do not attach to mouse fibroblast monolayers possessing the appropriate K/D antigens. This is in contrast to studies by Brondz *et al*. (113) who showed that CTLs are capable of being absorbed onto fibroblast monolayers, possessing the appropriate K/D antigens. In addition, cytotoxic T cells directed at the K/D antigens are found in the spleen (Tables 20 and 21) and this would indicate these cells are already present within the spleen and have not

been filtered out in the periphery. Thus, the lack of proliferative reactivity against the class I molecules must have a different basis.

Another in vivo environmental effect is evident when a comparison between H-2 and I region GVH reactions are compared. Entire H-2 mismatched reactions produce many primed cells, 2×10^6 to 5×10^6 cells/spleen, while I region or K/D mismatched reactions only yield about one sixth of the number of primed cells (Table 4). In addition, the quality of the primed cell reactivity in I region GVH is substantially weaker than the H-2 primed cells, despite the fact that the same number of cells were used to initiate the GVHR as well as used in the PLT assays. In contrast, the MLR system predicts both reactions will yield large numbers of primed cells which will respond very well in PLT assays. This anamolous finding indicates some in vivo synergistic effect is occurring in the entire H-2 GVHR, while in the I region GVHR an essential component is either diminished or is missing. The same type of finding is also observed in the Mls minor histocompatibility systems. In vitro reactions against the Mls determinants are very strong and demonstrate excellant secondary responsiveness, similar to I region mismatches. Primed cells generated in minor histocompatibility GVH reactions fail to show strong responsiveness (Tables 16 and 17).

In K/D disparate reactions, no proliferative responses are demonstrated (Table 15), as would be predicted from the data observed in the entire H-2 mismatched reactions (Tables 6,7,10,11,12,13,14). For some unknown reason these GVH primed cells do not respond in the same way as do the in vitro primed cells. In primary MLRs K/D disparate combinations are very strong. It would appear that either the in vivo environment does not permit proliferation towards K/D antigens or that in vivo restraints placed upon these reactions are not present in the in vitro conditions. The former possibility is probably correct because if the GVH primed cells derived from an entire H-2 mismatch are restimulated twice in vitro, the pattern of restimulation that is observed is identical to that pattern seen when the GVH primed cells are first tested. No additional reactivity is generated against the class I molecules, indicating no new antigens are recognized de novo.

What the mechanism of restraint is, remains only speculative. Perhaps, the crowded environment of the spleen, lymph node or liver does not allow the lymphoblasts to expand to the same extent as they do in the free environment of a tissue culture flask. In the flask conditions, the primed lymphocytes probably keep expanding until optimal or maximum cell concentrations are reached. It should be noted

that the primed cells obtained from GVH animals on days 5 to 14 of the reaction appear to be in a primed state and do not show any residual mitotic activity (as determined by ^{3}H-TdR incorporation by primed cells alone). When MLR reactive cells are tested too early without allowing sufficient time for the primed cells to revert back into small lymphocytes, background ^{3}H-TdR incorporation is very high and frequently masks any secondary responses which may be present. From this observation it is postulated that the GVH primed cells have matured quicker and have reached an equilibrium. These endstage cells do not need to replicate anymore and now these cells only mediate their effects without further need for cell division. This explanation could explain why proliferative responses to K/D antigens are not seen. In vivo the CTL precursors proliferate quickly in response to K/D antigens and this intermediate step is greatly shortened or lost, while in MLR conditions this intermediary stage has been prolonged indefinitely due to the lack of the in vivo restraints.

Cytotoxic T cells are also readily apparent in entire H-2 disparate reactions (Tables 20, 21), supporting previously published data (114). The presence of CTLs in tissue other than lymphoid e.g. liver (Table 21), is a new finding and does fit with the current dogma concerning GVHD

pathogenesis. Although CTLs are seen transiently in K/D disparate reactions, present in the spleens on day 7 of the GVHR but not by day 22, no CTL activity is recoverable when (Table 22) these animals are dying with GVHD (Figure 5). The presence of transient CTL activity does agree with a previous study by Hamilton and coworkers (115,116), who have suggested that CTL precursors do emigrate from the lymphoid tissue when they become active CTLs. Their studies showed that CTL precursors are present in the spleens of animals who are not displaying clinical signs of GVHD. However, when GVHD does start presenting itself clinically, CTL activity is readily apparent. Little if any CTL activity is detectable in I region GVHs (Table 25), but was demonstrated in the minor histocompatibility reaction of CBA/Ca anti-AKR (Table 32).

When purified exogenous interleukin 2 was added *in vivo* to a K/D disparate combination, the mortality rate sharply increased and this rate approached that of an entire H-2 mismatch GVH (Figures 5 and 20). Interestingly, CTL activity in the spleens of these animals did not increase to the extent as would be predicted by CTL activity found in the entire H-2 disparate GVH. Instead, CTL activity that is observed is identical to the amount of CTL activity found in the normal K/D disparate GVHR (Tables 23 and 24). These data

support Hamiliton and Parkman's premise that mature CTLs could have migrated out of the spleen.

These IL 2 studies do not prove that CTL activity is responsible for the mortality that is observed. Other possible mechanisms of IL 2 activity exist such as: increased natural killer cell activity, increased helper T cell activity or activation of other leukocytes which trigger an inflammatory response. IL 2 has been reported to enhance NK activity in vitro (117) as well as help maintain helper T cells in vitro both in human and murine systems (118,119). Soluble T cell derived factors, possibly IL 2 are known to cause mast cells to degranulate (120), leading to a histamine release which is an early part of the hypersenitivity response.

The presence of undetectable CTLs or CTL precursors which are expanded in vitro by IL 2 does seem to correlate with mortality fairly well. In an H-2 mismatch, the presence of large numbers of CTLs in the spleen and liver correlates well with the rapid demise of the hosts by days 5 to 14. The large number of CTLs no doubt plays a role in the host's death. In the B10.AQR anti-(B10.T(6R) x B10.AQR)F_1 combination these expanded lines lyse the host's I-A^q positive cells, but the vast majority of the cytotoxic T cells appear to recognize the donor cells as being foreign (Tables 26 and 28). Thus, a host versus graft reaction also

appears to be a possible mechanism of the GVH process. Even though these hosts are nearly lethally irradiated (650r) the host is still able to mount some type of counter reaction. It should be noted that studies performed by Dr. A. Kimura (personal communication) have shown that even in 550r sublethally irradiated mice some form of cytotoxic cells can be generated. The histopathological lesions of GVH are identical to HVG (104), so the pathology does not reveal any clear distinctions. By the very nature of this reaction it is impossible to distinguish whether the donor or host cells are becoming the effector cells. Since both strains of mice possess the identical K^q and D^d molecules it would be impossible to distinguish donor cell from host cell by normal serological tests. In addition, the activated cells are T cells and they would not be expected to bear Ia markers in sufficient quantity to distinguish it from the other. Even if Ia markers were detected by very sensitive methods such as flow cytometry, the intrepretation would be questionable because Delovitch et al. (121) have reported that donor T cells are capable of absorbing the host's Ia molecules during GVH reactions.

One interesting finding of the previous combination is that the reaction of B10.AQR anti-B10.T(6R) is very weak in its ability to generate CTLs or CTLp, while the reverse reaction, B10.T(6R) anti-B10.AQR is a very strong reaction.

In fact, this reaction is even able to overcome a sublethal dose of radiation to develop CTLs. This observation sheds light on a previously reported anomaly first described by Elkins (122), which was not readily explainable at the time its publication. In this system, Elkins demonstrated that when newborn B10.AQR mice were injected with B10.T(6R) cells the majority of these mice died, while in the reverse direction, B10.T(6R) mice completely survived an injection of B10.AQR cells. Since the newborn mice are totally immunoincompetent at birth and are unable to mount any kind of reaction, only GVH reactions occur. Thus, only those reactions in the hosts which develop sufficient numbers of CTLs or CTLp will develop mortality. From the polarity of the reaction in developing CTL activity in vitro with IL 2 it is apparent that B10.T(6R) cells should readily develop cytotoxic activity against B10.AQR, but the reverse reaction is significantly weaker. Thus, Elkin's data can now be explained in terms of CTL activity in vivo.

In another I region mismatch, (B10 x B10.Q)F_1 anti-B10.MBR rapid mortality was observed (Figure 23). The time in which mortality was observed was identical to that corresponding to a whole H-2 mismatch (B10 x B10.Q)F_1 anti-B10.BR. When these GVH splenic cells are expanded with IL 2 in vitro for 10 days, both GVH and HVG reactions are present with about equal cytotoxic strengths (Table 29).

This case is slightly different from the previous situation, where HVG was considerably stronger than GVH.

In contrast to the two previous I region combinations, the (B10.MBR x B10.GD)F_1 anti-B10 combination produces a survival curve with 30% mortality (Figure 24), which indicates a weak GVHR. When these splenocytes from day 5 of the GVHR are expanded in vitro with IL 2, only a weak cytolytic response can be generated against the host (Table 30). Such a weak cytotoxic response could conceivably give rise to a weak GVHR. From the strength of this in vitro response it would appear that proliferative types of cells outnumber the CTL precursors and thus limit the amount of IL 2 that the CTLp could absorb. It would therefore be of importance to clone these cells so that a homogenous cell line could be obtained to demonstrate strong cytolytic activity.

In the B10.S(9R) anti-(A.TL x B10.HTT)F_1 combination, no CTL or CTLp activity is demonstrable (Table 34). Furthermore, no mortality is detected even after 40 days after engraftment (Figure 25). This shows that the lack of CTL activity matches the lack of GVHD mortality.

In the minor histocompatibility combinations examined, cytotoxic T cells are demonstrated in those combinations in which mortality is seen. In CBA/Ca anti-AKR, cytotoxic T cells can be found which lyse AKR cells strongly, and DBA/1

and DBA/2 cells weakly (Table 32). The survival curves of such reactions reveal incomplete mortality (Figure 27). In another combination, BALB/c anti-DBA/2, no cytotoxic effectors can be found in the animals on the days they were tested (Tables 33 and 34). But if cells from a BALB/c mutant strain, BALB/c^{dm2}, (a strain which lacks the L^d molecule), are injected into DBA/2 mice, a completely different survival profile is obtained (Figure 26). This curve is one in which death occurs by day 12. Cytotoxic cells directed at the L chain have been reported (123) previously and it appears that in this GVH reaction CTLs may again play a major role in GVHD pathogenesis.

In summary, these series of experiments provide a correlation between GVH mortality and the presence of cytotoxic T cell activity. These data tend to favor previous theories about in vivo graft rejection, whether it be skin, kidney or graft versus host; indicating that cytotoxic T cells play a role in tissue destruction. Recently, these theories have come under attack by several groups including McKenzie et al. (124,125) and Mason (126,127). Their major arguments have relied on the phenotype of the cells recovered from graft rejection sites along with the corresponding negative CML assays. Because they have eliminated Lyt 2+ cells from their initial donor populations and fail to find subsequently Lyt 2+ cells or cytotoxic

activity, they believe that no CTLs are present in their systems. In countering such studies it must be pointed out that cytotoxic cells do not necessarily have to be Lyt 2+. Swain et al.(47) have shown that killer cells towards I region antigens are Lyt 1+ 2- cells. In the present study the B10.AQR anti-(B10.T(6R) x B10.AQR)F_1 effector cells generated were largely Lyt 1+ (80%) while some cells are weakly Lyt 2+ (15 to 20%). Furthermore, McKenzie and coworkers did not expand their recovered cell populations in vitro with IL 2 as done here and firmly rule out cytotoxic T cell potential. The data presented here, then, would tend to agree with the findings that when GVH primed cells are tested in CML assays the same day as they are removed from the host, there does not appear to be any anti host CTL activity. But what if McKenzie and fellow workers had expanded their recovered cells with IL 2?

These studies simply correlate GVHD mortality with the presence of CTL or CTLp. They do not show the mechanism through which they are capable of working. The CTL or CTLp may expand and attack the host's leukocytes or bone marrow cells leaving the host temporarily immunoincompetent and thereby susceptible to infections. In H-2 disparate GVH combinations the large number of CTLs probably kill the epithelial cells lining the gut and the hepatocytes which leads to quick mortality. However, in combinations with

slower mortalities the CTLs probably do not attack the intestine and liver, but are more active against target cells with high amounts of K/D antigens, such as leukocytes.

4.4 The Nonspecific Factors Influencing Mortality in GVH

Besides the specific functional immunological reactions which have been discussed, there also exists nonspecific factors which could play a significant role in the pathogenesis of GVHD. Inflammatory cells most noticeably the polymorphonuclear leukocytes were always obtained from effected GVH tissue, i.e. spleen, lymph nodes and liver. Fifty percent of the recovered leukocytes in the entire H-2 GVH organs were PMNs, while in K/D or I region GVHD, 90% of these populations were PMNs. The presence of these cells could be explained two ways. First, the natural occurrence of PMNs: is that hematopoeisis in the mouse can occur in the spleen while the liver can be a site of extramedullary hematopoeisis (128). Second, the activated lymphocytes could be secreting chemotactic factors or growth factors, such as interleukin 3 (IL 3). These factors would attract or stimulate colonies of granulocytes to develop in situ. IL 3 has been reported to be secreted by T cells/cytotoxic T cells (129). In addition, IL 3 has been reported to contain granulocyte colony stimulating factors (130) so this

possibility of PMNs being attracted to the GVH effected organs is indeed feasible. Irradiated control mice do show the presence of granulocytes, but the quantities of these cells (0.05×10^6 cells/spleen) is never as great as those seen in the GVH reactions (5.0×10^6 cells/spleen). Also, the lymph nodes are not sites of hematopoeisis, yet PMNs are found here, thus, the second possibility could be responsible for this phenomenon in the GVH lymph nodes.

What ever the reason PMNs are present, the question still remains: do the PMNs play any role in the associated tissue destruction? From the 24 and 48 hr CML assays, it would appear that PMNs are not directly lytic, despite the prolonged CML assays (Table 34). However, from the liver histology, it is noticeable that the PMNs frequently have invaded the parenchyma along with the lymphocytes. The PMNs could be attracted into such areas by the T cells. Because the PMNs have such a short lifespan, these cells may not exit from this sites, but die and release their lysosomal enzymes. These enzymes could then exacerbate and accelerate any tissue destruction that is actively occurring. This model would support McKenzie's idea of a delayed hypersensitivity response occurring in graft rejection sites (124,125). This mechanism also tends to be supported by the indirect evidence seen in the human clinical situation, that when GVHD occurs it is frequently alleviated with

immunosuppressive drugs such as cortisone and steroids, which are also known to inhibit inflammatory reactions (131).

Another apparently nonspecific mechanism important in GVHD pathogenesis is depicted when primed T lymphocytes induce mortality in sublethally irradiated syngeneic, allogeneic or third party hosts. The pathology of these lesions in syngeneic mice are markedly different from the typical GVH reaction. The spleens of these animals are often packed with lymphocytes (Figure 33), while the livers of such animals are often congested and edematous. Isolated patches of cellular infiltrates with associated mild necrosis are found. Although there is no perivascular cuffing of leukocytes, these infiltrates are markedly distinct (Figures 34 and 35) from those seen in a normal GVHR (Figure 10). In contrast, the lesions found in allogeneic animals reconstituted with primed lymphocytes are similar to classical GVHD (Figure 29). The spleens are necrotic, the livers have infiltrates in them with associated coagulative type necrosis.

It has been reported that donor T cells circulate in the course of GVHD. Studies by Sprent and Miller (132,133,134,135) have shown that donor cells on days 1 and 2 are primarily found in the spleen and lymph nodes of the host and that by day 4 blast cells can be collected from the

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thoraic duct. This finding demonstrates that primed cells traffic through the host during the course of GVHD and are not merely sessile.

The ability of primed cells to mediate GVHD has also been observed in this study (Figures 28 and 36). These cells were primed against the host's histocompatibility antigens, prior to engraftment, and as such, probably are responsible for the tissue destruction that is seen. However, this assumption may not be correct, since primed helper T cell clones and lines known not to possess any cytotoxic potential are also capable of precipitating GVHD (Figure 36). Again the lesions appear similar to what is seen in GVHD caused by injecting virgin allogeneic splenocytes. The helper clones and lines are directed at the I-A^q molecule and this reaction is analogous to the B10.AQR anti-(B10.T(6R) x B10.AQR)F_1 reaction. The mortality induced by the clones may be due to the proliferation of clones and associated graft versus host reaction via a hypersensitivity reaction. Evidence favoring this explanation is that a heteroclitic clone (clone 4 - RY1/Y2) is least effective in generating mortality. The low responsiveness of this clone in vitro towards the I-A^q molecule could conceivably delay development of GVHD. While those strongly in vitro proliferative clones would mediate quicker GVHD. The other possible explanation is that the host may be responding

against the graft as was observed in the B10.AQR anti-(B10.T(6R) x B10.AQR)F_1 reaction previously. Finally, a third possibility could be that the donor cells respond against the host and produce the necessary interleukins to maintain the host cells responding against the graft. Thus, it is still premature to conclude which reaction GVH or HVG is directly responsible for the mortality which is seen in this situation.

4.5 The Attempts to Prevent GVHD Using Anti-Host I-A Antibody

From the PLT of GVH primed cells it has been demonstrated that the I-A molecule is responsible for the major source of donor cell proliferation which is observed in vitro. Experiments using anti host I-A antisera to inhibit subsequent recognition of the host's I-A antigen, thereby preventing donor cell recognition proved unsuccessful in this model.

Possible explanations for this failure include 1) not all the antigenic sites were covered, and 2) the antibody was internalized and removed from the cell surface allowing the donor lymphocytes to recognize the host's I-A molecules. Obviously different protocols need to be studied to

determine the efficacy of using anti-I-A antisera. This approach was not pursued.

4.6 The Attempts to Prevent GVHD using Newborn Suppressor Cells

From the work of Peeler et al.(106) a suppressor cell and its associated factor have been described which operates by inhibiting in vitro MLR and CML reactions. It was therefore of interest to determine whether these suppressor entities would function in vivo by preventing GVHD.

CBA/J ($H\text{-}2^k$) newborn splenocytes are removed from one to four day old neonates, have the capacity to prevent GVHD by donor B10.BR ($H\text{-}2^k$) cells in (B10 x B10.BR)F_1 mice (Tables 35 and 36).This was not so when adult CBA/J splenocytes are cultured with the B10.BR cells, which resulted in no inhibition of GVHD. Thus, the ability of CBA/J newborn cells to exert some type of GVHD suppression eliminates the possibility that the B10.BR cells had simply become primed towards CBA/J determinants and therefore lacked the ability to respond towards the $H\text{-}2^b$ antigens.

Those animals which received the B10.BR cells treated with or without the CBA/J adult cells developed the classical GVHD syndrome: diarrhea, weight loss, lethargy etc. within the normal time frame. Most of the animals which received the B10.BR cells treated with the CBA/J newborn

cells did not show signs of GVHD at the time when the control host animals were dying. When these treated mice were dissected at day 15, leukocytic infiltrates were common in the liver. Perivascular cuffing was also prominent. The parenchyma was however intact and presented no cellular destruction, totally unlike previous GVHR where leukocytic infiltrates proved destructive to the parenchyma. The intestines were also affected, but not to the extent that was observed in the control GVHR. About one fourth of the villi were dilated and devoid of leukocytes, the rest of the villi appeared normal. In control GVH animals, nearly 95% of the villi were effected. Thus, the experimental animals did not appear to be wasting by the associated malabsorption problems. The leukocytes recovered from the spleen proved to be equivalent with the number of leukocytes recovered from the GVHD suppressed mice demonstrated little or no functional activity as compared to the reactivities seen by normal GVH primed cells.

As these acute GVHD suppressed reactions proceed, the animals begin to degenerate by day 25. The mice begin to develop skin lesions: loss of hair and redness of the skin being common. Mononuclear cell infiltrates are found in the dermis (Figure 38). The liver also appears to be effected. Histologically the liver is completely destroyed, the parenchyma is "punched out", only nuclear remnants are left

(Figures 39 and 40). No cell boundaries are visibly detectable. However, the intestines appear normal. When the spleens are removed, the number of recovered leukocytes was the same as day matched irradiated control mice, and these cells demonstrated no proliferative activity towards the antigens syngeneic to the host (Table 38). Furthermore when these cells are expanded with IL 2 for one week in vitro these cells exhibited no CTL activity (Table 40). Instead, a granulocyte/basophil like cell appear in culture (Figure 41). The presence of these cells was never experienced before using the IL 2 in vitro expansion technique. The presence of these cells was also noted when the spleens were freshly prepared, so it does not appear such cells developed de novo from other cells. Whether these cells are responsible for the chronic GVHD seen is not apparent at this time.

To determine if the CBA/J newborn cells had incapitated the adult B10.BR cells, the B10.BR cells were tested for mitogenic responses using Con A and LPS (Table 37). The newborn cells themselves were unable to respond to Con A and LPS. The B10.BR cells treated overnight with the newborn CBA/J cells were still quite capable of responding to the T and B cell mitogens. This experiment establishes that the B10.BR cells treated with the CBA/J newborn cells have not been killed or have been functionally inactivated.

There does appear to be genetic restrictions in what types of GVHD the CBA/J newborn cells are capable of suppressing. When B6 splenocytes were incubated with the newborn CBA/J cells, these treated cells were not capable of suppressing GVHD against B10.WB, another entire H-2 mismatched reaction (Table 41). Neither of these two mouse strains possess any of the histocompatibility antigens derived from the $H\text{-}2^k$ haplotype. This might suggest that the donor cells must share I regions with the suppressing CBA/J for GVHD suppression to occur.

Another experiment (Table 41) using CBA/J newborn cells demonstrated that B10.A(5R) and B10.A(3R) cells are also capable of being suppressed enough to prevent acute GVHD in sublethally irradiated B10 mice. This experiment therefore correlates the $I\text{-}E^k$ molecule with the CBA/J mediated GVHD suppression. Whereas, a shared $I\text{-}A^k$ molecule with the CBA/J cells does not appear to be sufficient for this suppression to occur. Unfortunately, this hypothesis does not hold when other mouse strains are studied. For example, B10.A(2R), B10.MBR, and B10.HTT which possess the $I\text{-}E^k$ were not capable of being suppressed by the CBA/J newborn cells. Therefore, this restriction is unable to be explained at the present time.

Another study demonstrated that newborn splenocyte mediated GVHD suppression can occur in the $H\text{-}2^d$ system

(Table 41). In this combination newborn mice from either DBA/2 or SEA were capable of suppressing B10.D2 splenocytes from causing acute GVHD in B6 mice. Quite unexpectedly newborn splenocytes from BALB/c and SEC mice failed to suppress GVHD from occurring.

To determine whether any newborn spleen cell preparation would demonstrate GVHD suppression, newborn (B10.BR x SWR)F_1 splenocytes were used in place of the CBA/J newborn cells. As demonstrated in Table 35 no suppression of GVHD in (B10 x B10.BR)F_1 mice was induced, despite the fact that the same protocols and techniques were employed. To eliminate the possibility that the H-2^q haplotype interferred with the priming reaction newborn (B10.BR x SWR)F_1 cells were incubated with (B10.BR x SWR)F_1 adult cells overnight and then injected into (B10 x B10.BR)F_1 mice. Again the same negative results were observed: no GVHD suppression was observed.

These previous studies have indicated that cellular interactions, most probably within the I region are needed before GVHD suppression can be effected. When newborn spleen cell supernates are used they do in fact inhibit MLR primary reactions irregardless of the genetic disparity. And these factors only marginally prevent GVHD from occuring in (BALB/c x B6)F_1 mice reconstituted with BALB/c cells

(Figure 46). This argues that some unknown cellular interactions are probably responsible for GVHD suppression.

The newborn suppressor factors can be fractionated into 2 populations: one with a molecular weight less than 10,000 daltons and the other with a molecular weight greater than 10,000 daltons. The mode of action of these substances appears to be different. The time of addition study revealed that addition of the low molecular weight substance could inhibit a primary MLR even up to 72 hrs, right before the ^{3}H-TdR was added to the culture. This low molecular weight substance probably contains either: histamine, thymidine or prostaglandins which simply reduce ^{3}H-TdR incorporation nonspecifically. However, histamine's activities could be eliminated because it was unable to inhibit thymidine incorporation in mixed lymphocyte reactions. Prostaglandin E_2 did suppress the mixed lymphocyte reactions, but this substance was only effective if it was added within the first 48 hours of the reaction, so this factor is not likely to be the low molecular weight factor. In contrast, the higher molecular weight substance only suppresses the primary MLR within the first 48 hours of the reaction. The size profile of the suppressor factor reveals that the higher molecular weight substance probably contains 2 entities (Figure 42); one with a higher molecular weight

a molecular weight roughly the size of albumin. This substance is not alpha fetoprotein, because when anti-alpha fetoprotein antibody is tested in a gel diffusion test, no lines of identity form with purified alpha fetoprotein (Figure 44).

Previous works (88,89,136) have used newborn suppressor cells to inhibit local GVH assays in adult F_1 animals as well as temporary suppression of systemic GVHD in 5 to 7 day old mice. Interestingly, the newborn splenocytes which were successfully used in such assays have come from CBA, AKR and A/J mice. These reports have shown that neonatal spleen cells or newborn splenocytes from 1 to 3 day old mice have the ability to suppress H-2 mismatched GVH reactions as measured by footpad swelling or splenomegaly methods. However these suppressor cells are short lived, since mice older than 5 days do not possess suppressor activity. Other studies have demonstrated that newborn splenocytes secrete suppressor factors which inhibit MLRs, CTL development and T cell mediated antibody synthesis (137,138,139,140). These workers also claim that suppressor factor synthesis declines in the newborn mice around days 4 to 7 like the GVH suppression observed by Ptak and Skowron-Cendrzak (88,89). Research by Arygris (141) has claimed that the newborn suppressor factors can be distinguished into 2 fractions,

one with a molecular weight less than 10,000 daltons and the other factor with a molecular weight greater than 10,000 daltons. However, the higher molecular weight factor was thought to be simply dimers or multiples of the lower weight fragments. These factors were also thought to be secreted by newborn T cells. In contrast, previous work by Peck and fellow workers (106,139) have associated the source of these factors to monocytes and mast cells. Whereas, this work does not address this particular controversy, the findings reported here confirm that newborn splenocytes secrete soluble factors which are capable of suppressing primary MLRs (142,143). Additionally, this work also demonstrates that newborn suppressor cells are capable of suppressing acute lethal GVHD in adult mice.

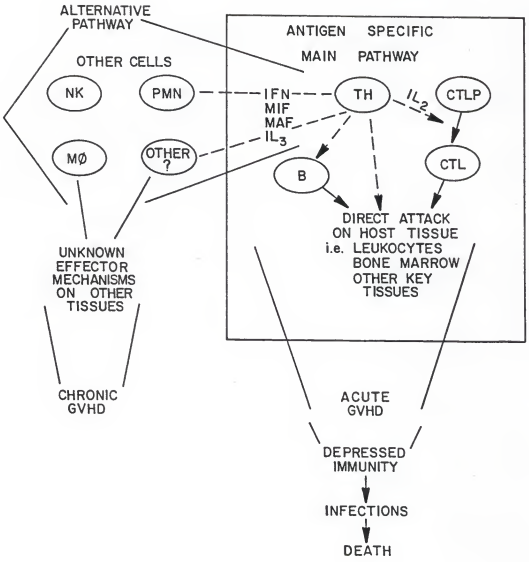
One point worth noting has been the correlation of suppression with Mls^a positive mice. The exact relationship and role of the Mls positive cells remains only speculative. First, the Mls locus or a gene linked to the Mls may control the type or the amount of suppressor factor(s) that are secreted. Newborn cells constantly secrete factors and this continous supply of factor provides the stimulus for GVHD suppression. Second, recognition of the Mls antigen does not lead to any harmful effect, but activates the suppressor limb of the immune system. Previous

work by Festenstein (100) has shown that the Mls antigens do not behave the same way as the H-2I antigens do. For example Table 1 illustrates that I region antigens induce good splenomegaly and produce good stimulation indices, nevertheless, Festenstein has shown that Mls disparate combinations do not induce splenomegaly but instead induce good in vitro reactivity. Cytotoxic T cells and specific antisera have never been produced against the Mls antigen, despite numerous unreported attempts. Follow up studies have shown that in vivo CTL generating reactions involving H-2 and Mls disparate reactions produce fewer CTLs than do the comparable H-2 disparate reactions (144). Likewise, Arygris (145) has found that when DBA/2 cells or P-815 cells (a mastocytoma derived from DBA/2) are injected into B6 mice, suppressor cells and suppressor factors are induced both in vitro and in vivo. These suppressor entities are able to inhibit MLRs and CTL development. Similar unreported findings by Peck testify to the uniqueness of this Mls disparate reaction. Finally, in a different experimental setting, Click has reported that Mls disparate marrow grafts survive much better even in H-2 mismatches (146).

Whatever the mechanism of suppression is, one thing that remains is that adult mice reconstituted with allogeneic cells treated with CBA/J and SEA newborn splenocytes do develop chronic GVHD. With the exception of Rappaport et al's combination: B10.D2 anti-(DBA/2 x B10.D2)F_1 (96), no reported study examined to date has described chronic GVHD in the mouse system. Sprent and Korngold have recently speculated that both chronic and acute GVHD are caused by T cells (147,148,149). The evidence supporting the cause of acute GVHD is fairly solid, in that treatment of lymphocytes with anti-Thy antisera plus complement eliminates all types of GVH which would have appeared, both local and systemic. The evidence linking chronic GVHD is not so well delineated. This research has showed that T cell reactivity may be completely abolished (Tables 38 and 40) or working in such low basal levels that the normal acute GVHD effector mechanisms may not be working properly and that another route of GVHD effectors may be working in its place (Figure 47). These other effectors do not have to be lymphocytes, perhaps the inflammatory cells such as PMNs and monocytes may be the cells mediating this form of the disease. If this other effector limb is indeed working then the lesions which are observed do not have to be identical to those produced by the T cell mediated effector mechanism(s). Thus, this disease process which is

observed here could then be more comparable to the situation in the human bone marrow transplantation setting where chronic GVHD occurs. Thus, this proposed model would also explain why a form of GVHD occurs in the humans undergoing bone marrow transplantation, even after the donor T cells have been eliminated from the reconstituting cell populations.

Figure 45. Proposed model for GVH morbidity.


ALTERNATIVE PATHWAY
ANTIGEN SPECIFIC MAIN PATHWAY
OTHER CELLS
NK
PMN
IFN
MIF
MAF
IL3
TH
IL2
CTLP
MØ
OTHER ?
B
CTL
DIRECT ATTACK ON HOST TISSUE i.e. LEUKOCYTES BONE MARROW OTHER KEY TISSUES
UNKNOWN EFFECTOR MECHANISMS ON OTHER TISSUES
CHRONIC GVHD
ACUTE GVHD
DEPRESSED IMMUNITY
INFECTIONS
DEATH

REFERENCES

1. Elkins, W.L. Progress in Allergy. 15:78. 1971.

2. Grebe, S.C. and Streilein, J.W. Adv. Immunol.22:118. 1976.

3. Klein, J. Biology of the Mouse Histocompatibilty Complex. Springer-Verlag. New York. 1975.

4. Hood, L.E., Weissman,I.L. and Wood, W.B. Immunology. Benjamin/Cummings. Reading, Mass. 1978.

5. Hellstrom, I., Hellstrom, K. and Allison, A. Nature. 230:49. 1971.

6. Cohen I. and Wekerle H. in Autoimmunity. editor: N. Talal. Academic Press. New York. 1977.

7. Gershon, R., Cohen, P. and Liebikaber, S. J. Immunol. 105:586. 1971.

8. Mills, V. Orbach-Arbouys, S. and Gershon, R. J Immunol. 114:200. 1975.

9. Cerrotini, J., Nordin, A. and Brunner, K.T. J Exp. Med. 134:553. 1971.

10. Goldstein, P., Wigzell, H., Blomgren, H. and Snedmyr, E. J. Exp. Med. 135:890. 1972.

11. Emerson, E.E. and Thursh, D. J. Exp. Med. 137:1293. 1973.

12. Atkins, R.C. and Ford, W.L. J. Exp. Med. 141:664. 1975.

13. Ford, W.L., Simmonds, S. and Atkins, R.C. J. Exp. Med. 141:681. 1975.

14. Rolstad, B. Transplantation. 21:117. 1976.

15. Lankford, J., Blackstock, R., Skyatalowicz, V. and Hyde, R. Cell Immunol. 14:163. 1974.

16. Katz, D. Lymphocyte Differentiation. Academic Press. New York. 1976.

17. Makinodan, T., Santos, G. and Quinn, R. Pharm. Rev. 22:189. 1970.

18. Weston, W.L., Claman, N., and Kreuger, G. J. Immunol. 110:880. 1973.

19. Turk, J.L., Parker, D. and Poulter, L. Immunology. 23:493. 1972.

20. Hess, A.D. and Tutsuska, P. J. Immunol. 124:2601. 1980.

21. Larsson, E.L. J. Immunol. 124:2828. 1980.

22. Maugh, T. Science. 210:44. 1980.

23. Kunkel, A. and Klaus, G.G. J. Immunol. 125:2526. 1980.

24. Swain, S. and Dutton, R. J. Immunol. 124:437. 1980.

25. Rich, R.R. and Rich, S.S. J. Immunol. 114:1112. 1975.

26. Rich, S.S. and Rich, R.R. J. Exp. Med. 140:1588. 1974.

27. Sell, S. Ann. Intern. Med. 71:177. 1969.

28. Reinherz, E. and Schlossman, S.F. Cell. 19:821. 1980.

29. Reinherz, E., Kung, P., Goldstein, G., Levey, R. and Schlossman, S.F. PNAS. (USA).77:1588. 1980.

30. Katz, D., Graves, M., Dorf, M., Dimenzo, H. and Benacerraf, B. J. Exp. Med. 141:263. 1975.

31. Erb, P. and Feldmann, M. J. Exp. Med. 141:460. 1975.

32. Taussig, M., Isac, M. and Mozes, E. J. Exp. Med. 140:301. 1974.

33. Mozes, E., Isac, R. and Taussig, M. J. Exp. Med. 141:703. 1975.

34. Takemori, T. and Tada, T. J. Exp. Med. 142:1241. 1975.

35. Rich, R. and Pierce C. J. Immunol. 112:1360. 1974.

36. Bach, F., Widmer, M., Bach, ML. and Klein, J. J. Exp. Med. 136:1430. 1972.

37. Klein, J. and Park, J.M. J. Exp. Med. 137:1213. 1973.

38. Ford, W.L. Handbook of Experimental Immunology. Blackwell Scientific. London. 1975.

39. Klein, J. and Schreffler, D. J. Exp. Med. 135:924. 1972.

40. Demant, P. Folia Biol. (Praha) 16:273. 1970.

41. Tsoi, M.S, Storb, R., Dobbs, S., Meadill, A. and Thomas, E.D. J. Immunol. 125:2258. 1976.

42. Hayry, P. and Defendi, V. Science. 168:133. 1970.

43. Hayry, P. Andersson, L.C., Nordling, S. and Virolainen, M. Transplant. Rev. 12:91. 1972.

44. Andersson, L.C. and Hayry, P. Transplant. Rev. 25:121. 1975.

45. Kisielow, P. Nature. 253:219. 1975.

46. Shihu, H., Kisielow, P., Bean, M., Takahashi, T., Boyse, E.A. and Oettgen, H. J. Exp. Med. 141:227. 1975.

47. Swain, S.L., Dennert, G., Wormsley, S. and Dutton, R.W. Eur. J. Immunol. 11:175. 1981.

48. Herzenberg, L.A. and Ledbetter, J. (personal communication to Becton-Dickinson) 1980.

49. Cantor, H. and Boyse, E.A. J. Exp. Med. 141:1376. 1975.

50. Bach, F.H., Bach, M.L., Sondel, P. and Sunderadas, G. Transplant. Rev. 12:30. 1972.

51. Swain, S. and Panfili, P.R. J. Immunol. 122:383. 1979.

52. Hayry, P. Immunogenetics. 3:417. 1976.

53. Peck, A.B. and Wigzell, H. Immunogenetics. 6:529. 1978.

54. Peck, A.B., Klein, J. and Wigzell, H. J. Immunol. 125:1978. 1980.

55. Gillis, S. and Smith, K. Nature. 268:154. 1977

56. Baker, P.S., Gillis, S. and Smith, K. J. Exp. Med. 149:273. 1979.

57. Dennert, G. Nature. 277:476. 1979.

58. Glasebrook, A. and Fitch, F. Nature. 278:171. 1979.

59. Glasebrook, A. and Fitch, F. J. Exp. Med. 151:876. 1980.

60. Swain, S.L. and Dutton, R.W. J. Immunol. 124:437. 1980.

61. Watson, J. J. Exp. Med. 150:1510. 1979.

62. Watson, J., Aarden, L., Shaw, J. and Paetkau, V. J. Immunol. 122:1633. 1979.

63. Bonnard, G.D., Yasake, K. and Maca, R. Cell Immunol. 51:390. 1980.

64. Farrar, J., Mizel, S., Fuller-Farrar, J., Farrar, W. and Hilfiker, M. J. Immunol. 125:793. 1980.

65. David, T., Yu, Y., Chiorazzi, N. and Kunkel, H. Cell Immunol. 50:305. 1980.

66. Okada, M. and Henney C.S. J. Immunol. 125:850. 1980.

67. Scott, J.W., Ponzio, N., Orosz, C. and Finke, J. J. Immunol. 124:2378. 1980.

68. Elliott, B., Kimura, A., Peck, A.B., Nagy, Z. and Wigzell, H. Immunogenetics. 12:525. 1981.

69. Tees, R. and Schreier, M. Nature. 283:780. 1980

70. Taussig, M., Corvalin, J., Binns, R., Roser, B. and Holliman, A. Eur. J. Immunol. 9:768. 1979.

71. Tada, T. and Okumura, K. Adv. Immunol. 28:1. 1979.

72. Taniguiche, M., Takei, I. and Tada, T. Nature. 283: 227. 1980.

73. Eschar, Z., Apte, R., Lowry, I., Ben-Nariah, Y., Givol, P. and Mozes, E. Nature. 286:270. 1980.

74. Dennert, G. and Raschke, W. Eur. J. Immunol. 7:352. 1977.

75. von Boehmer, H., Hengartner, H., Nabholty, H., Lernhardt, W., Schreier, M. and Haas. W. Eur. J. Immunol. 9:592. 1979.

76. Sherman, L. J. Exp. Med. 151:1386. 1980.

77. Sredni, B., Tse, H. and Schwartz, R. Nature. 283:581. 1980.

78. Sredni, B., and Schwartz, R. Nature. 287:855. 1980.

79. Fathman, C.G. and Hengartner, H. Nature. 272:617. 1978.

80. Fathman, C.G. and Hengartner, H. PNAS. (USA). 76:5863. 1979.

81. Peck, A.B., Murgita, R. and Wigzell, H. J. Exp. Med. 147:667. 1978.

82. Peck, A.B., Murgita, R. and Wigzell, H. J. Exp. Med. 148:360. 1978.

83. Murgita, R., Goidl, E., Kontainen, S., Beverly, P.C. and Wigzell, H. PNAS. (USA). 75:2897. 1978.

84. Murgita, R. and Tomasi, T. J. Exp. Med. 141:269. 1975.

85. Murgita, R. and Tomasi, T. J. Exp. Med. 141;440. 1975.

86. Lau, L. and Linkins, S. Am. J. Obstet. Gynecol. 124: 533. 1976.

87. Peck, A.B., Murgita, R. and Wigzell, H. J. Immunol. 128:1134. 1982.

88. Skowron-Cendrzak, A. and Ptak, W. Eur. J. Immunol. 6: 451. 1976.

89. Ptak, W. and Skowron-Cendrzak, A. Transplantation. 24:45. 1977.

90. Globerson, A. and Umiel, T. Transplantation. 26:438. 1978.

91. Peck, A.B. and Bach, F. J. Immunol. Methods. 3:147. 1973.

92. Peck, A.B. and Click, R. Eur. J. Immunol. 3:385. 1973.

93. Peck, A.B. and Wigzell, H. Immunogenetics. 6:529. 1978.

94. Peck, A.B. and Bach, F. Scand. J. Immunol. 4:53. 1975.

95. Shimuzu, S., Konaka, Y. and Smith, R.T. J. Exp. Med. 152:1436. 1981.

96. Rappaport, H., Khalil, A., Halle-Pannenko, O., Pritchard, L. and Mathe, G. Am. J.Path. 96:121. 1979.

97. Festenstein, H. Transplant Rev. 3:74. 1970.

98. Festenstein, H. Transplant Rev. 15:62. 1973.

99. Festenstein, H. Transplant Proc. 8:339. 1970.

100. Molnar-Kimber, K. and Sprent, J. J. Exp. Med. 15:407. 1981.

101. Molnar-Kimber, K. and Sprent, J. Transplantion. 31:376. 1981.

102. Nakayana, E., Shiku, H., Stockert, E., Oettgen, H.F. and Old, L.J. PNAS. (USA). 76:1977. 1979.

103. Shinohara, N. and Sachs, D.H. J. Exp. Med. 150:432. 1979.

104. Hard, R.C. Transplantation. 17:613. 1974.

105. Peck, A.B., Smith, R.T. and Jadus, M.R. (submitted for publication) J. Immunol. 1982.

106. Peeler, K.L., Wigzell, H. and Peck, A.B. Scand. J. Immunol. (in press). 1982.

107. Wagner, H., Hardt, C., Heeg, K., Pfizenmaier, K., Stotter, H. and Rollinghoff, M. Immunobiol. 161:139. 1982.

108. Hardt, C., Rollinghoff, M., PFizenmaier, K., Mosman, H. and Wagner, H. J. Exp. Med. 154:262. 1981.

109. Bach, F.H., Bock, H., Graupner, K., Day, E and Klosterman, H. PNAS. (USA) 62:377. 1969.

110. Wolters, E.A.J. and Benner, R. Transplantation. 26:40. 1978.

111. Wolters, E.A.J. and Benner, R. Nature. 279:642. 1979.

112. Ford, W.L. and Gowans, J.L. Sem. Haematol. 6:67. 1973.

113. Brondz, B.D., Andreev, A.V., Egorova, S.G. and Drizlikh, G.I. Scand. J. Immunol. 10:195. 1979.

114. Cerrotini, J.C. and Brunner, K.T. Adv. Immunol. 18:67. 1974.

115. Hamilton, B.L., Bevan, M.J. and Parkman, R. J. Immunol. 126:621. 1981.

116. Hamilton, B.L. and Parkman, R. J. Immunol. 128:376. 1982.

117. Henney, C.S., Kuribayashi, K., Kern, D.E. and Gillis, S. Nature. 291:335. 1981.

118. Gillis, S., Smith, K.A. and Watson, J. J.Immunol. 124: 1254. 1980.

119. Watson, J., Gillis, J., Marbrook, D., Mochizuki, D. and Smith, K.A. J. Exp. Med. 150:849. 1979.

120. Nabel, G., Galli, S.J., Dvorak, H.F. and Cantor, H. Nature. 291:332. 1981.

121. Delovitch, T.L., Harris, J.F., Battistella, R. and Kaufman, K. J. Exp. Med. 155:61. 1982.

122. Elkins, W.L. Transplant. Proc. 8:343. 1976.

123. Levy, R.B. and Hansen, T.H. Immunogenetics. 10:7. 1982.

124. Loveland, B.E. and McKenzie, I.F.C. Transplantaion. 33: 217. 1982.

125. Loveland, B.E., Hogarth, P.M., Ceredig, R. and McKenzie, I.F.C. J. Exp. Med. 153:1044. 1981.

126. Mason, D.W. Transplantation. 32:222. 1981.

127. Dallman, M.J. and Mason, D.W. Transplantation. 33:221. 1982.

128. de Sousa, M. Lymphocyte Circulation. Academic Press. New York. 1982.

129. Ihle, J., Rebar, L., Keller, J., Lee, J.C and Hapel, A. Immunol. Rev. 63:5. 1982.

130. Garland, J.M. Immunology Today. 3:208. 1982.

131. Fauci, A.S. Am. J. Med. 64:729. 1978.

132. Sprent, J. and Miller, J.F.A.P. Cell. Immunol. 3:361. 1972.

133. Sprent, J. and Miller, J.F.A.P. Cell. Immunol. 3:385. 1972.

134. Sprent, J. and Miller, J.F.A.P. Cell. Immunol. 3:213. 1972.

135. Sprent, J. and Miller, J.F.A.P. J. Exp. Med. 139:1. 1974.

136. Rybcznska, Z., Sajak, A. and Skowron-Cendrzak, A. Arch. Immunol. Et Therap. Experiment. 29:341. 1981.

137. Mosier, D.E. and Johnson, B.M. J. Exp. Med. 141:216. 1975.

138. Basset, T.M., Coons, T.A., Goldberg, E.H. and Williams, J. J. Immunol. 119:1855. 1977.

139. Rodrigeuz, G., Andersson, G., Wigzell, H. and Peck, A.B. Eur. J. Immunol. 9:737, 1979.

140. Pavia, C.S. and Stites, D.P. Cell. Immunol. 42:48. 1978.

141. Arygris, B. Cell. Immunol. 62:412. 1981

142. Arygris, B. Cell. Immunol. 66:352. 1982

143. Arygris, B. Transplantation. 31:334. 1981

144. Matossian-Rogers, A. and Festenstein, H. J. Exp. Med. 143:456. 1976.

145. Arygris, B. Cell. Immunol. 57:62. 1981.

146. Click, R.E. Tranplant. Proc. 11:490. 1979.

147. Sprent, J. J. Exp. Med. 148:478. 1978.

148. Korngold, R. and Sprent, J. J. Exp. Med. 151:1114. 1980.

149. Sprent, J. and Korngold, R. Immunology Today. 2:189. 1981.

BIOGRAPHICAL SKETCH

Martin Jadus was born on January 10, 1953 in Girardville, Pennsylvania. He lived in Wilmington, Delaware until he graduated from the University of Delaware with Bachelor of Science Degrees in Biological Sciences and Chemistry in 1976. From September 1976 to August 1978 he lived in Melbourne Florida and received a Master of Science Degree in the Biological Sciences from Florida Institute of Technology. From 1978 until 1983 he attended the University of Florida. At present he plans to either move to the West Coast or to Uppsala, Sweden in order to continue active research in Immunology.

I certify that I have read this study and that in my opinion it confirms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it confirms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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